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(54) Title: COMPOSITIONS AND METHODS FOR INHIBITING XENOGRAFT REJECTION

(57) Abstract

The present invention provides compositions and methods useful to inhibit antibody-mediated xenograft rejection by xenogeneic transplant recipients. Accordingly, new methods of transplanting xenografts are also provided. Also provided are compositions which induce antibody-mediated xenograft rejection. Methods for isolating antigen expressed by endothelial cells of a xenograft and which induces antibody-mediated rejection of xenografts are also provided.

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# COMPOSITIONS AND METHODS FOR INHIBITING XENOGRAFT REJECTION

## I. BACKGROUND OF THE INVENTION

Organ transplantation is becoming an increasingly effective medical therapy for the long-term treatment of many otherwise fatal diseases. Prior to 1980, the major limitation to routine transplantation as a surgical procedure was that of organ rejection. In recent years, however, the development of powerful new immunosuppressive drugs has significantly improved organ graft survival. The major factor currently acting to prevent those who need a transplant from getting one is an acute shortage of human donor organs. Those who do receive transplants are having to wait longer to find a suitable match, compromising their chances for optimum results, and sometimes, survival. This shortage of donor tissue limits the application of transplantation to a small proportion of patients who could be expected to benefit from the operation and produces higher health care costs because of the requirement for continued intensive care of patients with chronic, terminal diseases.

One practical solution to the shortage of human donors is the use of organs from non-human donors. The exchange of tissues between two different species ("xenogeneic transplant"), however, results in a rejection reaction that is more aggressive than that observed for the transplantation of tissue between members of the same species ("allogenic transplant"). In addition, this reaction to xenografts has not responded well to the traditional immunosuppressive treatment which is effective to prevent loss of allograft, providing a clear suggestion that the immunologic mechanism of rejection is different in xenogeneic transplants than it is in allogenic transplants.

A failure to completely understand the immune mechanisms responsible for the rejection of xenografts has inhibited the development of more effective treatment regimens.

### A. The Rejection of Xenogeneic Transplants Is Characterized as Hyperacute or Accelerated

Transplantation is by definition the placement of foreign tissue ("donor") into a host recipient, thus an immunological attack on the foreign tissue ("rejection") is to be expected. The vigor with which this immunological assault is mounted has been correlated with the genetic disparity of the donor/recipient species. The greater the genetic disparity, the swifter and more acute the immunological rejection. Accordingly, the rejection of xenografts is typically more vigorous than allografts. Xenograft rejections have been classified into two general categories on the basis of this genetic disparity of the donor/recipient species and the rapidity of the graft rejection as compared to allograft rejection untreated recipients.

#### 1. Hyperacute Rejection is Characteristic of Transplants Between Discordant Species

In general, recipients of xenografts from genetically distant ("discordant") donors react by mounting a rapid, hyperacute rejection of the foreign graft. This form of rejection is seen with pig-to-human xenogeneic transplants and is associated with the loss of the xenograft within a few minutes or hours. This type of xenograft rejection has proven to be more difficult to control therapeutically than the accelerated rejection characteristic of xenogeneic transplants between concordant species, discussed below.

Therefore, these species combinations have been less attractive as potential organ donors for humans.

Generally, the literature reports two hypothesis for the pathogenesis of the hyperacute rejection. Some suggest that anti-xenograft antibodies are produced by the recipient prior to the xenogeneic transplant. These preformed antibodies are believed to precipitate the violent, hyperacute rejection of the xenograft as a result of the immediate and diffuse deposition of these antibodies in the graft and activation of the complement/coagulation systems. The other commonly held hypothesis is that the alternative complement pathway is directly activated by antigens expressed by the xenograft in some donor/recipient species combinations. Although there is some evidence in the literature to support each of these hypothesis, none of the evidence has proven sufficient to provide meaningful prevention or intervention in the immune response which results in hyperacute rejection of xenografts.

2. Accelerated Rejection is Characteristic of Xenografts Between Concordant Species

In contrast, grafts that are exchanged between more closely-related ("concordant") species are rejected in an accelerated, but not immediate, reaction. This form of rejection is seen with baboon-to-human xenogeneic transplants and is associated with the loss of the xenograft within a few (2-4) days of transplant.

Accelerated reactions were originally considered to represent an aggressive form of acute allograft rejection directed at a different species. See, Calne R Y., Transplant. Proc. 2:550-553 (1970). However, it has recently been reported that the accelerated rejection of xenografts is due to an antibody-mediated rejection of the donor graft that requires a period of a few days to result

in sufficient levels of antibody to cause the graft to fail. See, Cosenza et al., J. Heart Lung Transplant, 13:489-497 (1994).

Humans, for example, do not exhibit high levels of anti-xenograft antibodies to closely-related species, such as baboons and other non-human primates, prior to a xenogeneic transplant. Thus, these species have served as the preferred donors of xenograft for human. The implicit assumption is that the low level of preformed anti-xenograft antibodies in a concordant reaction leads to the development of a rejection reaction that may be more easily managed with traditional forms of immunosuppressive therapy. Although the rejection of the xenograft is mediated by the rapid production of anti-xenograft antibodies post-transplant, there is a small window of opportunity to intervene in the immune response before the rejection of the xenograft is complete.

### 3. Comparative Pathogenesis of the Hyperacute and Accelerated Rejection of Xenogeneic Tissue

Rejection of both discordant and concordant xenografts is associated with evidence of vascular damage, rather than the extensive accumulation of inflammatory cells that is characteristic of T cell-mediated reactions. In those cases of xenogeneic transplants into humans or experimental animals when sequential examination has been conducted, the pathological changes present in the xenografts are consistent with antibody-mediated damage to the vessels of the grafts. See, Rosengard, B.R., et al. J. Heart Transplant, 5:263-266 (1986); Bailey, L. et al. J. Am. Med., 254:3321 (1985); Bogman, M.J.J.T., et al., Am.J.Pathol., 100:727-735 (1980).; Linn, B.S., et al., Transplant. Proc., 3:527 (1971); Ertel, W. et al., Transplant. Proc., 16:1259-

1261 (1984). The primary pathological changes include endothelial cell swelling, necrosis, interstitial edema, platelet and fibrin thrombi, and hemorrhage. See, Xenotransplantation. The Transplantation of Organs and Tissue Between Species, eds. Cooper, D.K.C., Kemp, E., Reemtsma, K., and White, D.J.G. Berlin:Springer-Verlag, 1991, p. 181-242.

Although the exact nature of the target antigens and the humoral response that mediate accelerated and hyperacute rejection reactions have largely been unknown, including whether the anti-xenograft antibodies are polyclonal or polyspecific, the changes are compatible with antibody binding to antigens expressed on the endothelium of the donor graft vessels and activation of the complement cascade. In the case of hyperacute rejection of xenografts, the antibodies are apparently immediately available to mount an attack which results in rejection. While, in the case of the accelerated rejection of xenografts, the antibodies which mediate rejection must first be manufactured.

### B. A Reliable Model for Xenogeneic Transplant Rejection Is the Hamster-to-rat Transplant Model

Examination of the rejection of heart xenografts between different rodent species has demonstrated that the accelerated rejection of hamster hearts by rat recipients is a reliable model of the accelerated pattern of rejection characteristic of baboon-to-human xenogeneic transplants. See, Cramer, D.V. et al., Transplant.Proc.,25:2864-2847 (1993); Makowka, L. and Cramer, D.V., "The use of xenografts in experimental transplantation." In: Handbook of Animal Models in Transplant Research, eds. Cramer, D.V. Podesta, L. and Makowka, L. Boca Raton: CRC Press, Inc. 1993, p. 299-310. Hamster heart xenografts are rejected by naive rats within about four days due the rapid rise in anti-xenograft

IgM antibodies and the humoral destruction of the graft. See, Wu G. D., et al., Transplant. Proc., 24:691-692 (1992). In this rodent species combination, like the baboon-to-human primate species combination, preformed anti-xenograft antibodies appear to be present at low levels in the serum of the recipient prior to transplantation, but are apparently not in sufficient number to produce a hyperacute response. Antibodies produced by the rat after transplant with hamster tissue display a pattern of reaction with heart membrane antigens in Western blots that is similar to that seen with the preformed antibodies. See, Cramer D. V., et al., Transplantation 54:403-408 (1992).

The primary difference between the accelerated rejection reaction occurring in this hamster-to-rat model and the hyperacute rejection reaction observed in pig-to-human xenogeneic transplants appears to be quantitative rather than qualitative: the time period required for low levels of preformed anti-xenograft antibodies in the rat recipient to rise to sufficient levels to cause the loss of the hamster xenograft. As discussed above, rejection of both hyperacute and delayed xenografts is associated with evidence of humoral vascular damage, rather than the extensive accumulation of inflammatory cells that is characteristic of T cell-mediated reactions.

Specie combinations that normally are characterized by an accelerated rejection reactions can also be shown to display hyperacute rejection following either sensitization of the recipient with donor tissue immunogen prior to transplant or by passive transfer of hyperimmune sera prior to transplant. Immunizing recipient rats with hamster lymphocytes or hamster cardiac grafts prior to a hamster-to-rat xenogeneic transplant results in an antibody-mediated hyperacute rejection of hamster xenografts. These hamster tissue immunogens reportedly stimulate a rapid rise in the

normally low levels of IgM antibody that react with hamster lymphocytes and vascular endothelium and graft rejection follows. Likewise, passive transfer of serum from a rat that has rejected a hamster xenograft to a naive rat results in hyperacute rejection of a hamster xenograft by the naive rat. See e.g., Wu, G.D., et al., Transplant Proc. 24:691 (1992).

There are, as described in a variety of current reviews, apparently other important components of the xenografts rejection reactions, including, for example, direct activation of the alternative complement pathway, modification of the rejection reaction by regulatory complement proteins following antibody binding, and the contribution of the cellular immune response. See, e.g., Auchincloss, H., Jr. Transplantation 46:1-20 (1988); Xenotransplantation. The Transplantation of Organs and Tissue Between Species, eds. Cooper, D.K.C., Kemp, E., Reemtsma, K., and White, D.J.G. Berlin:Springer-Verlag, 1991, p. 69-79; Advances in Transplantation, eds. Hackel, B. and AuBochon, J., Bethesda, Maryland: Amer. Assoc. Blood Banks, 1993, p. 93-112; Johnston P. S., et al., Transplant. Proc. 23:877-879 (1991). While each of these pathogenetic mechanisms may be important in some or all xenograft rejection reactions, it is abundantly clear that the deleterious activity of preformed antibodies against the xenograft must be controlled, preferably without debilitating the entire immune system, if xenografts are to provide a meaningful solution to the problems associated with allograft transplants. Accordingly, there has existed a need for methods and compositions to inhibit antibody-mediated rejection of xenografts by recipient animals.

## II. BRIEF DESCRIPTION OF THE INVENTION

The present invention provides novel and powerful immunological compositions and methods for inhibiting antibody-mediated rejection of xenografts by a transplant recipient. Through pre-transplant treatment of the xenograft with these immunological compositions, preferably supplemented by post-transplant therapeutic treatment of the xenograft recipient with these immunological compositions and, optionally, chemical immunosuppressive agents, prolonged survival of the graft can be achieved, providing the first, critical step to long-term xenograft survival. This ability to intervene in the immune response to xenogeneic tissue opens the door to the use of xenografts as a meaningful alternative to the shortage of available allogeneic organs.

Thus in accordance with the present invention there is provided methods of inhibiting rejection of a donor xenograft by a recipient animal, comprising modifying antigen expressed by cells of the xenograft, without causing lysis of the cells, to inhibit binding of recipient anti-donor xenograft antibody to said antigen, wherein said antigen present in unmodified form induces an antibody-mediated immune response in the recipient animal. A preferred method of modifying such antigen, particularly those expressed by endothelial cells of the xenograft, comprises contacting non-lytic, anti-donor xenograft antibody material with said antigen for a time, at a temperature, and at a pH suitable to bind the antibody material to the antigen. Anti-donor xenograft antibody material also provided by the present invention is characterized as immunoreactive with antigen expressed by endothelial cells of the xenograft and capable of inhibiting

antibody-mediated rejection of the xenograft by a recipient animal.

This invention also provided, for the first time, monoclonal antibodies that immunoreact with antigen expressed by endothelial cells of donor xenografts and which are capable of inducing antibody-mediated rejection of the xenograft. These monoclonal antibodies, as well as the polypeptides from which they are formed and the polynucleotides which encode them, will provide researchers with powerful and reliable reagents greatly needed in the search to better understand and control the xenogeneic transplant rejection reaction.

The present invention also provides methods of using the compositions of the present invention to isolate and further characterize the antigen(s) responsible for precipitating xenogeneic transplant rejection reaction.

## III. BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is the nucleotide sequence of the variable heavy chain region of the rat anti-hamster xenograft monoclonal antibody designated HAR-1. The sequence of the SAX oligonucleotide has been artificially included with the 5' end of the sequence. The 5' untranslated region appears in lower case. The boundaries for the framework and CDR regions are deduced from conventions estabilished by Kabat and Wu for mouse and human immunoglobulin and are indicated above the sequence. Primers which were used to generate this and other sequences are labeled and designated by underline.

Figure 2 shows the cDNA nucleotide sequence of the variable heavy chain segment of the rat anti-hamster xenograft monoclonal antibody designated HAR-1 aligned with its germ-line counterpart VH1.1 obtained by genomic amplification of newborn LEW rat liver DNA. The two

sequences are 99.2% (351/354 nucleotides) homologous. HAR-1 cDNA sequence differs at three nucleotides from the germ-line sequence. The first difference leads to a replacement Leu by Val in the leader sequence whereas the 2 others are "silent". Intron 1 untranscribed region is presented in lower case. "*" indicates identity.

Figure 3 shows the cDNA nucleotide sequences of the variable heavy chain region of the rat anti-porcine xenograft monoclonal antibodies designated HA75DBF1 and IH21H7 aligned to demonstrate sequence homology. "*" indicates identity.

Figure 4 shows the cDNA nucleotide sequence of the variable heavy chain segment of the rat anti-pig xenograft monoclonal antibody designated HA75DBF1 aligned with its germ-line counterpart VHRAP.1a obtained by genomic amplification of newborn LEW rat liver DNA. The two sequences are 98.6% (286/290) homologous. HA75DBF1 cDNA differs at three nucleotides from the germ-line sequence. All three of these differences occur in the framework regions and are indicated by a box. "1" indicates identity.

## IV. DETAILED DESCRIPTION OF THE INVENTION

The use of organ transplants for the treatment of end-stage diseases has become an established and highly effective therapeutic regimen. The success of organ transplantation, however, has resulted in a shortage of human donor organs creating a major limitation to the more widespread use of this technology. One of the practical solutions to the shortage of human donors is the use of species other than humans for organ donation. Even if only on a short-term basis, such organs could provide temporary life-support until a more suitable organ became available. However, the exchange of tissues between two different

species ("xenogeneic transplant") results in a rejection reaction that is more aggressive than that observed for the transplantation of tissue between members of the same species ("allogeneic transplant"). Although the rapidity of rejection in a xenogeneic transplant can be somewhat lessened (accelerated instead of hyperacute) by selecting the donor from a concordant species, there still remains a significant risk of transmitting potentially serious pathogens to the recipient when xenogeneic transplant is performed between concordant species. These xenograft rejection reaction have not responded to traditional immunosuppressive treatment and the lack of understanding of the immune mechanisms responsible for the loss of the graft has inhibited the development of more effective treatment regimens.

The present invention provides novel and powerful immunological compositions and methods for inhibiting the antibody-mediated component of the rejection of xenografts. Through pre-transplant treatment of the xenograft with these immunological compositions, preferably supplemented by post-transplant therapeutic treatment of the xenograft recipient with these immunological compositions, prolonged survival of the graft can be achieved, providing the first, critical step to long-term xenograft survival. This ability to intervene in the immune response opens the door to the use of xenografts as a meaningful alternative to the shortage of available allogeneic organs.

This invention also provided, for the first time, monoclonal antibodies that immunoreact with antigen expressed by endothelial cells of donor xenografts and which are capable of inducing antibody-mediated rejection of the xenograft. These monoclonal antibodies, as well as the polypeptides from which they are formed and the polynucleotides which encode them, are valuable tools in the

hands of researches who seek to better understand the xenogeneic rejection reaction and to devise new compositions and methods to overcome it, but whose efforts have been frustrated by the lack of available and reliable reagents with which to conduct their work.

Another highlight of the present invention are methods of using the compositions of the present invention to isolate and further characterize the antigen(s) responsible for precipitating xenogeneic transplant rejection.

A. Anti-Donor Xenograft Antibodies

In accordance with the present invention there is provided isolated and substantially purified anti-donor xenograft antibody that is immunoreactive with antigen expressed by endothelial cells of a xenograft from a donor animal and that are capable of inducing antibody-mediated rejection of the xenograft by a recipient animal.

As used herein the terms "isolated," "substantially pure," or "recombinant" in their various grammatical forms as a modifier of proteins including antibodies and antibody materials, polypeptides, amino acid sequences, polynucleotides, and nucleic acid sequences or molecules means that the proteins, polypeptides, amino acid sequences, polynucleotides, and nucleic acid sequences or molecules so designated have been produced in such form by the hand of man, and thus are separated from their native *in vivo* cellular environment. As a result of this human intervention, the isolated, pure and/or recombinant, proteins, polypeptides, amino acid sequences, polynucleotides, and nucleic acid sequences or molecules of the invention can be produced in large quantities and are useful in ways that the proteins, polypeptides, amino acid

sequences, polynucleotides, and nucleic acid sequences or molecules as they naturally occur are not.

The terms "antibody" and "antibody molecule" in their various grammatical forms are used herein as collective nouns to refer to a population of immunoglobulin molecules which may be polyclonal or, more preferably, monoclonal in origin and which may be of any isotype, preferably of the IgM isotype.

The term "immunoreact" in its various grammatical forms means specific binding between an antigenic determinant-containing molecule, such as an antigen, and a molecule containing an antibody combining site such as an antibody molecule or antibody material.

As used herein the term "xenograft" refers to grafted tissue or tissue intended for use in a transplant operation between animals, including humans, that has been derived from a donor animal that is a different species than that of the recipient animal or intended recipient animal of the tissue. Typically, the tissue is organized in the form of a critical body organ. A variety of xenografts suitable for use in the present invention are well-known in the art, such as kidney, heart, liver, lung, pancreas, and the like.

The term "donor animal" as used herein is a collective noun referring to the species of animal from which the xenograft is taken for transplant and can include, for example, domesticated animals such as pigs, non-human primates such as baboons, rodents such as hamsters and rabbits, and the like. Accordingly, the xenograft, being tissue of the donor animal, may be further designated herein by the type (species) of donor animal from which the xenograft originates, e.g. hamster xenograft.

The term "recipient animal" is used herein as a collective noun referring to the species of animal which

receives the xenograft and includes, for example, humans, domesticated animals, primates, rodents, and the like.

As used herein the phrase "antibody-mediated rejection of the xenograft" refers to an immunological attack on the xenograft which is driven by humoral, as opposed to cell specific immunity, and which is thus mediated by antibody molecules. Antibody-mediated rejection of a xenograft can be accelerated or hyperacute. Preferably, anti-donor xenograft antibody of the present invention is capable of inducing hyperacute rejection of the xenograft.

Anti-donor xenograft antibodies of either monoclonal or polyclonal form can be produced using techniques presently known in the art. For example, polyclonal and monoclonal antibodies can be produced as described, for example, in Harlow and Lane, Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory 1988), which is incorporated herein by reference. Altered antibodies, such as chimeric, humanized, CDR-grafted or bifunctional antibodies can also be produced by methods well known to those skilled in the art. Such antibodies can also be produced by hybridoma, chemical or recombinant methodology described, for example in Ausubel et al., Current Protocols in Molecular Biology (Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. 1993), also incorporated herein by reference. Exemplary methods of making and isolating monoclonal anti-donor xenograft antibodies are provided in EXAMPLES below.

The phrase "polyclonal antibody" in its various grammatical forms refers to a population of antibody molecules that contains more than one species of idiotope capable of immunoreacting with epitopes on a particular antigen. Polyclonal antibody of the present invention specifically includes a mixture of more than one monoclonal antibody that immunoreact with different epitopes on the same antigen.

The phrase "monoclonal antibody" in its various grammatical forms refers to a population of antibody molecules that contain only one species of idiotope capable of immunoreacting with a particular epitope on an antigen. A monoclonal antibody typically displays a single binding affinity for an epitope with which it immunoreacts; however, a monoclonal antibody may be a molecule having a plurality of idiotopes, each immunospecific for a different epitope, e.g., a bispecific monoclonal antibody.

Monoclonal antibodies are typically composed of antibodies produced by clones of a single cell called a hybridoma that secretes (produces) but one kind of antibody molecule. In accordance with the present invention hybridomas capable of producing antibodies and antibody materials having specific immunoreactivity with antigen expressed by endothelial cells of a xenograft from a donor animal is provided and described in greater detail below. One of skill in the art will recognize that the hybridomas disclosed herein can be used to produce other immortal cell lines that produce antibody and antibody material of the present invention.

A hybridoma cell is formed by fusing an antibody-producing cell and a myeloma or other self-perpetuating cell line. The preparation of such hybridomas was first described by Kohler and Milstein, Nature, 256:495-497 (1975), which description is incorporated by reference. Polypeptide-induced hybridoma technology is also described by Niman et al., Proc. Natl. Sci., U.S.A., 80:4949-4953 (1983), which description is also incorporated herein by reference.

To obtain an antibody-producing cell for fusion with an immortalized cell, an animal can either be transplanted with a xenograft from a donor animal or inoculated with a xenogeneic immunogen. If the transplant technique is used,

preferably, the animal receiving the transplant is of the same species as the recipient animal.

The term "xenogeneic immunogen" in its various grammatical forms is used herein to describe a composition containing donor endothelial cell antigen as an active ingredient used for the preparation of the antibodies against antigen expressed by endothelial cells of a xenograft from a donor animal. The amount of immunogen used to inoculate the mammal should be sufficient to induce an immune response to the immunizing antigen. This amount depends, among other things, on the species of animal inoculated, the body weight of the animal, the source and form of the donor endothelium antigen in the immunogen, and the chosen inoculation regimen as is well known in the art.

Antibody-producing cells, e.g. splenic lymphocytes, are harvested from the immunized animal, or the transplanted animal after rejection of the xenograft, and can be fused with myeloma cells using polyethylene glycol ("PEG"). Fused hybrids are selected by their sensitivity to hypoxanthine, aminopterin and thymidine selection medium ("HAT").

A monoclonal antibody of the present invention can also be produced by initiating a monoclonal hybridoma culture comprising a nutrient medium containing a hybridoma that secretes anti-donor xenograft antibody molecules. The culture is maintained under conditions and for a time period sufficient for the hybridoma to secrete the antibody molecules into the medium. The antibody-containing medium is then collected. The antibody molecules can then be further isolated by well known techniques.

Other methods of producing a monoclonal antibody, a hybridoma cell, or a hybridoma cell culture are also well known. See, for example, the method of isolating monoclonal antibodies from an immunological repertoire as described by Sastry et al., Proc. Natl. Acad. Sci., 86:5728-5732 (1989);

Huse et al., Science, 246:1275-1281 (1981); and International Patent Application No. PCT/US92/03091 all of which are incorporated herein by reference.

Anti-donor xenograft antibody can be identified by screening for the presence of antibody molecules that immunoreact with antigen expressed by endothelial cells of the donor animal and which are capable of inducing antibody-mediated rejection of the xenograft. Screening methods for such immunoreactivity can take any one of several commonly used immunoassay formats, including for example, radioimmunoassay (RIA), enzyme linked immunosorbent assay (ELISA) or immunofluorescent assay. The source of antigen in these immunoassays is endothelial cells or endothelial cell-containing tissue sections from a donor animal. More preferably, the source of antigen for the immunoassay is endothelial cells or endothelial cell-containing tissue sections from the same type of tissue as the xenograft and, most preferably, from the same individual animal that donates the xenograft. An antibody is considered to "immunoreact with antigen expressed by endothelial cells of the donor animal" when binding by the test antibody exceeds binding detected in the negative control by at least about two times, preferably by at least about 2.5 times, and even more preferably by about three times, particularly when flow cytometric assay is employed. The negative control for such assays is PBS or more preferably autologous serum. A presently preferred method for screening for immunoreactivity is described with greater detail in the flow cytometry assay provided in the EXAMPLES below.

Screening methods to identify antibodies having the ability to induce antibody-mediated rejection of the xenograft include *in vitro* cytotoxicity assays such as or example, flow cytometric cytotoxicity assay or MTT cytotoxicity assay, and *in vivo* rejection experiments using

animal models. In an *in vitro* format, antibody-mediated, complement-dependent cytotoxicity indicates that antibody tested has the ability to mediate rejection of the donor xenograft by the recipient animal. The source of antigen for the cytotoxicity assays can be as described above for the immunoreactivity assays, or other whole cells from the donor animal can be used. Preferably, the source of complement is animal serum which produces low background, e.g. Low Tox™ rabbit or mouse serum (Cedar Lane Laboratories, Hornby Ontario, Canada). Preferably, as measured in a flow cytometric cytotoxicity assay, antibody is capable of inducing antibody-mediated rejection of a xenograft by a recipient animal when more than about 20% cell death is detected. A presently preferred flow cytometric cytotoxicity assay for detecting the ability of antibody to induce antibody-mediated rejection of a xenograft by a recipient animal is described with greater detail in the EXAMPLES below. One of skill in the art will appreciate that the assay described in Examples can easily be adapted for specie combinations of interest.

Alternatively, *in vivo* rejection experiments can be employed, where appropriate, to detect the capability of antibody to induce antibody-mediated rejection of a xenograft by a recipient animal. First, the median survival times of a xenograft for the recipient animal must be characterized, preferably, the mean rejection time of a xenograft following pre-transplant passive transfer of hyperimmune serum to a recipient animal is also determined. A recipient animal then receives by passive transfer an inoculation of the putative antibody. The quantity of inoculum depends on the several factors such as the type and weight of the recipient animal. Transplant of the xenograft is performed on the pre-treated recipient and xenograft survival time is determined. Rejection is considered to

have occurred when the xenograft fails. An antibody is capable of inducing antibody-mediated rejection of the xenograft by the recipient if pre-transplant, passive transfer of the antibody results in accelerated, or more preferably hyperacute, rejection of a subsequently transplanted xenograft. Histological examination of the rejected xenograft can be performed to confirm that the rejection was antibody-mediated.

Since the hyperacute rejection of hamster tissue by sensitized rats and rats that have received passive transfer of hyperimmune serum are regarded as reliable models of the pattern of xenograft rejection in humans, these hamster-to-rat transplant models were used to generate anti-donor xenograft antibodies of the present invention and exemplify screening techniques.

1. Xenograft Rejection in the Rat Model

As a point of reference and as reported in Table 1, a series of experiments were conducted to quantify the characteristic allograft rejection in naive LEW rats which is generated by transplant between the same species ("ACI rat --> LEW rat"), the characteristic accelerated xenograft rejection in naive LEW rats which is generated by transplant between concordant species ("Hamster --> LEW rat"), the characteristic hyperacute xenograft rejection pattern in naive LEW rats which is generated by transplant between discordant species ("Guinea pig --> LEW rat"), and the characteristic hyperacute rejection pattern in LEW rats which is generated by passive transfer of hyperimmune rat serum followed by transplant between concordant species ("Hamster --> HRS+LEW rat"). The heterotopic cardiac transplantation procedure was followed for all transplants.

Table 1. Cardiac Xenograft Survival in Rat Model

| Group | N | Graft Survival (Mean ± 1 S.D.) |
|---|---|---|
| **Allograft-type Rejection** | | |
| ACI rat --> LEW rat | 5 | 7.0 ± 0.5 days |
| **Accelerated Rejection** | | |
| Hamster --> LEW rat | 5 | 3.9 ± 0.2 days |
| **Hyperacute Rejection** | | |
| Guinea pig --> LEW rat | 5 | 14.8 minutes |
| Hamster --> HRS + LEW rat | 5 | 14.0 minutes |

Naive LEW rats received passive transfer of hyperimmune rat serum by intravenous injection of about 0.1 to 0.5 ml sera. Rejection was considered to have occurred when the xenogeneic heart stopped beating.

"Hyperimmune serum," as that term is used herein, refers to sera from an animal that has rejected a xenograft originating from the donor animal. Preferably, the hyperimmune serum is from the same species of animal as the recipient animal on which the *in vivo* rejection experiment is to be performed. Hyperimmune serum may be further designated herein by the animal from which it was derived, e.g., hyperimmune "rat" serum.

2. The Hamster-to-Rat Model Mimics the Humoral Component of Xenograft Rejection in Humans

These transplant experiments also confirmed that the accelerated rejection by the rat of the hamster xenografts is closely associated with the production of rat anti-hamster IgM antibodies. Using an ELISA to detect the immunoreactivity of serum antibodies it was demonstrated that prior to transplantation, the serum of naive rats contained a small amount of preformed IgM antibody (detectable only at low dilutions, e.g., 1:2 to 1:4) that bound to the endothelium of the normal hamster heart and hamster lymphocytes. After transplantation, the total amount of serum IgM (but not IgG) antibody rose rapidly until rejection of the hamster heart xenograft at about Day 4 post-transplantation. Total IgM levels at about Day 4 post-transplant were ~ 2.4 mg/ml as quantified by immunoprecipitation and compared to standards. This rise in total IgM was paralleled by a rapid rise in the rat recipient serum of IgM antibody that reacts exclusively with the vascular endothelium of normal hamster hearts and normal hamster splenic lymphocytes.

In addition, the Western blot binding patterns conducted against hamster heart proteins using the pre- and post-transplant sera in accordance with the EXAMPLES below are strikingly similar. Serum from naive LEW rats detects multiple (>8) protein bands by Western blot analysis of hamster heart proteins. Rejection of the graft at 4 days post-transplant is associated with serum antibodies that recognize a similar pattern of Western blot protein bands, most with greater intensity, reflecting a higher level of antibody post-transplant, but with the same pattern of reactivity. In addition, histopathological lesions seen in the hamster-to-rat cardiac xenografts were consistent with an acute, IgM antibody-mediated vascular rejection.

3. Pre-transplant Treatment with Anti-donor Xenograft Monoclonal Antibody Induces Hyperacute Antibody-mediated Rejection

In accordance with a preferred embodiment of the present invention, IgM producing B-cells from the spleen of rats that had received hamster heart transplants were used to generate antibodies of the present invention. Rat hybridomas were produced by fusing rat myeloma cells (YB2/0) with splenic lymphocytes from LEW rat recipients of hamster cardiac xenografts. (See, EXAMPLES below.) The hybridomas were screened for IgM antibody production in an ELISA format as described in the EXAMPLES below. Antibodies from IgM-producing hybridomas were screened, using the immunofluorescent assay described in the EXAMPLES, for immunoreactivity to hamster endothelial antigens. Such antibodies were also screened for the ability to induce antibody-mediate hyperacute rejection of hamster heart xenografts in naive rats by *in vivo* rejection experiment. As determined by the preliminary rejection experiments described in Table 1 and used herein with regard to this animal model, the term "hyperacute rejection" refers to the rejection of a xenograft in less than about one hour.

Several hybridomas were created which produce anti-donor xenograft antibodies, or more specifically, rat anti-hamster xenograft monoclonal antibodies, including four hybridoma cell lines stored in liquid nitrogen by Dr. Donald Cramer in suite 250N of the Transplant Biology Research Laboratory of Cedars-Sinai Medical Center, located at 150 North Robertson Blvd., Beverly Hills, California, 90211. These three hybridoma cell lines are labeled and identified by the following laboratory names: HAR-1, ID12BF3, ID12CF2, and FC2EG11.

These rat anti-hamster xenograft monoclonal antibodies demonstrated binding to hamster endothelium derived from various tissue sources including such vascularized organs as the heart, kidney, gut, liver, lung, brain, and tongue, and more specifically including arterial and capillary endothelial cells, lymphatic endothelium, intestinal epithelium, thymic epithelium and aortic endothelium in tissue sections. The target antigen(s) are also associated with the reticular cells and macrophages of the splenic red pulp, the periarterial lymphoid sheath, and the thymic medullary epithelium.

Rat anti-hamster xenograft monoclonal antibodies also demonstrated a clear ability to induce antibody-mediated rejection of the xenograft by naive LEW rats. For example, when a one milliliter aliquot of culture supernatant from HAR-1 was passively transferred pre-transplant to naive LEW rats, the mean rejection time of the subsequent hamster

xenografts was 10 minutes (Table 2, Group "HAR-1"). Clearly, the rejection induced by HAR-1 is hyperacute and is mediated by binding to endothelial antigen, as was the rejections

Table 2. Passive Transfer of IgM Monoclonal Antibodies Having Specificity for Hamster Endothelium Induce Hyperacute Rejection Of Hamster Xenografts in Naive Rats

| Group | N | Survival Time | Median Survival Time |
|---|---|---|---|
| HAR-1 | 5 | 8, 9, 10, 10, 10 | 10 minutes |
| Poly | 5 | 10, 13, 15, 15, 18 | 15 minutes |
| 9D6 | 5 | 4, 4, 4, 4, 4 | 4 days |

induced by ID12BF3, ID12CF2 and FC2EG11 (median survival time of 30 minutes each). Naive rats receiving hamster cardiac xenografts and which are not subjected to pre-transplant treatment with HAR-1 have a mean rejection time of 3.9 days (Table 1, "Hamster --> LEW rat"). The mean graft rejection time induced by pre-transplant treatment of naive rats with culture supernatant from a hybridoma producing IgM monoclonal antibody that lacked binding specificity for endothelium (Table 2, "9D6") was four days, the time to rejection normally seen in naive rats. The hyperacute rejection precipitated by HAR-1 was slightly more

severe, but comparable to, the hyperacute rejection precipitated by pre-transplant treatment of naive rats with serum from a transplanted rat. (Table 2, Group "Poly").

HAR-1 binding specificity was further characterized in terms of its inability to bind tissue other than hamster endothelium. Flow cytometric analysis and hemagglutination assays of HAR-1 demonstrates limited binding of the antibody to erythrocytes or splenic lymphocytes.

Through histopathologic examination of tissue using immunohistochemistry to demonstrate antibody and complement binding and thrombosis in vessels (See EXAMPLES below.), it was determined that HAR-1 reacts specifically with vascular endothelium, triggering complement activation and intravascular thrombosis heart xenografts. The binding of the rat anti-donor xenograft monoclonal antibodies and the activation of complement with subsequent intravascular thrombosis are the same lesions as those seen in models of hyperacute rejection due to passive transfer of hyperimmune serum.

Finally, it was determined it was determined through Western blot analysis of hamster heart proteins that HAR-1 binds proteins producing 40 kDa and 80 kDa.

Thus, in accordance with more specific defined embodiments of the present invention there is provided isolated and substantially purified anti-donor xenograft antibody ("anti-hamster xenograft antibody") characterized as immunoreactive with antigen expressed by endothelial cells of a hamster xenograft and capable of inducing antibody-mediated rejection of the hamster xenograft by a recipient animal. Preferably, the hamster xenograft is heart tissue or the recipient animal is a rat. More preferable the hamster xenograft is heart tissue and the recipient animal is a rat. Although such antibodies may be of any isotype, preferably they are of the IgM isotype and

bind proteins producing 40 kDa and 80 kDa bands by Western blot analysis.

In yet another embodiment of the present invention, the isolated and substantially purified anti-hamster xenograft antibody is further characterized as comprising at least one polypeptide encoded by a nucleic acid sequence which includes a sequence having at least about 90% homology, preferably at least about 95% homology, more preferably at least about 98% homology, and most preferred about 100% homology to the sequence defined by nucleic acid residues 58 through 351 of SEQ ID NO: 1 or nucleic acid residues 168 through 440 of SEQ ID NO: 9.

One of skill in the art will appreciate that having provided the sequences of several anti-donor xenograft antibodies, polypeptides, and antibody materials of the present invention, additional embodiments of such compositions can be generated which have amino acid residue sequence substantially identical to a sequence specifically shown herein merely by making conservative substitutions in one or more residues of the sequence with a functionally similar residue and which displays the ability to mimic the compositions as described herein. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

The phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a

non-derivatized residue provided that such polypeptide displays the requisite binding activity.

"Chemical derivative" refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include, for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. Polypeptides of the present invention also include any polypeptide having one or more additions and/or deletions of residues relative to the sequence of a polypeptide whose sequence is shown herein, so long as the requisite activity is maintained.

In an still another embodiment of the rat anti-hamster xenograft antibody of the present invention, the antibody is characterized as being immunoreactive with antigen expressed by endothelial cells of a hamster xenograft, capable of inducing antibody-mediated rejection of the hamster xenograft by a recipient animal and comprising at least one polypeptide encoded by a nucleic acid sequence including the sequence defined by nucleic acid residues 58

through 420 of SEQ ID NO: 1 or by a nucleic acid sequence including the sequence defined by nucleic acid residues 1 through 354 of SEQ ID NO: 3. Preferably, the anti-hamster xenograft antibody further comprises at least one polypeptide encoded by a nucleic acid sequence including the sequence defined by SEQ ID NO: 5.

4. Rat Anti-porcine Xenograft Antibodies Demonstrate Similar Antigen Binding to Human Anti-porcine Xenograft Antibodies

Having demonstrated through the hamster-to-rat xenogeneic transplant model (1) that the accelerated rejection reaction is mediated by preformed anti-xenograft antibodies of the IgM isotype which are polyspecific for antigens of the vascular endothelium of the transplant tissue, and (2) that a hyperacute rejection reaction can be induced by anti-donor xenograft antibodies, monoclonal antibodies were then generated that exhibit binding specificity representative of the human anti-porcine xenograft antibodies which mediate the rejection of pig tissue by humans.

Humans who have not undergone xenogeneic transplant or immunization possess serum antibodies that are known to bind a broad range of xenogeneic cell surface-associated molecules including antigens present on erythrocytes, vascular endothelium, platelets, and lymphocytes in a variety of species, including pigs. Preformed human anti-porcine xenograft antibodies bind at least six antigens expressed by pig aortic endothelial cells ("PAEC") with molecular weights of 44 kDa, 80 kDa, 115kDa, 125 kDa, 135 kda and 200 kDa by Western blot analysis. Three antigens of similar molecular weights (115kD, 125kD, and 135kD) which are expressed on pig platelet cells are also bound by these

preformed human anti-porcine xenograft antibodies. In addition, these preformed human anti-porcine antibodies bind pig lymphocytes since absorption of human serum with pig lymphocytes removes the binding of these preformed antibodies to PAEC.

In general, the antibodies that appear to be most closely associated with the xenograft reaction are of the IgM isotype, although IgG and IgA antibodies have reported to be involved in the rejection of pig tissue by humans. However, the cytotoxic activity of human preformed antibodies to pig endothelium is most clearly the result of the binding of IgM and not IgG antibodies.

Similar to the rejection of pig xenografts by humans, hyperacute rejection of pig xenografts by rats is initiated by preformed IgM antibodies in the rat recipient's serum that bind to antigens expressed by the pig xenograft. Accordingly, a pig-to-rat model was employed to generate and characterize anti-porcine xenograft antibodies. A panel of 83 rat monoclonal antibodies to PAEC ("rat anti-porcine xenograft monoclonal antibodies") were generated using traditional hybridoma techniques as described in the EXAMPLES below and in Yokayama, W.M., Current Protocols in Immunology, Colifan, J, et al., (eds.). Greere Publishing of Wiley - Interscience, 1991, 2.5.4, incorporated herein by reference. Briefly, LEW rats were immunized with PAEC and splenic lymphocytes of the rat were harvested and fused with rat YB2/0 myeloma cells to produce hybridomas. Antibodies secreted by these hybridomas were then subjected to several assays including assays which identified antibodies which are immunoreactive with antigen expressed by endothelial cells of a xenograft from a donor animal and which are capable of inducing antibody-mediated rejection of the xenograft by a recipient animal.

Of the approximately 250 hybridomas generated and screened, 83 hybridomas were identified as secreting antibody having immunoreactivity with antigen expressed by PAEC. PAEC immunoreactivity was determined in an ELISA format as described in the EXAMPLES below using PBS and pig serum as negative controls.

Another selection criterion which is presently prefered is the ability of the rat anti-porcine xenograft monoclonal antibodies to immunoreact antigen expressed by pig platelets as is characteristically observed with preformed human anti-porcine xenograft antibodies. Using a flow cytometry assay, the panel of 83 rat anti-porcine xenograft monoclonal antibodies were tested for their ability to immunoreact with pig platelets. Most of these monoclonal antibodies tested positive. These results were confirmed in an ELISA assay using pig platelets as targets and human serum as a positive control.

Another selection criterion for anti-porcine xenograft antibody which is presently prefered is the IgM isotype. The isotypes of the monoclonal antibodies were determined in an ELISA format as described in the EXAMPLES below. Twelve of the 83 hybridomas having immunoreactivity for PAEC secrete antibody of the IgG isotype, and the remainder are IgM.

Anti-porcine xenograft monoclonal antibodies were identified amongst the 83 monoclonal antibodies which demostrated immunoreactivity with antigen expressed by PAEC by their ability to induce antibody-mediated rejection of the xenograft by a recipient animal. Accordingly, these 83 monoclonal antibodies were their ability to kill PAEC in a complement-mediated flow cytometric cytoxicity assay as described in the EXAMPLES below. Eleven anti-porcine xenograft monoclonal antibodies identified each being highly cytotoxic (>80%) to PAEC in the complement-mediated

cytotoxicity assays. The results were identical when purified rabbit complement or rat serum was used as a source of complement. Autologous complement, as expected, was not cytotoxic for PAEC.

Hybridoma cell lines secreting these anti-porcine xenograft antibodies are stored in liquid nitrogen by Dr. Donald Cramer in suite 250N of the Transplant Biology Research Laboratory of Cedars-Sinai Medical Center, located at 150 North Robertson Blvd., Beverly Hills, California, 90211. These hybridoma cell lines are labeled and identified by the following laboratory names: HA73C4; HA71G4; HA73D7; HA75D8; HA72G3; HA71E3; HA73HB; IE31D8; IG121H7; DA910E4; and IH21H7.

The present invention thus includes isolated and substantially purified anti-donor xenograft antibody ("anti-porcine xenograft antibody") characterized as being immunoreactive with antigen expressed by endothelial cells of a pig xenograft and capable of inducing antibody-mediated rejection of the pig xenograft by a recipient animal. In a particular embodiment of the invention, the pig xenograft is heart tissue or liver tissue. In another embodiment of the invention the recipient animal is a human.

Preferably, the anti-porcine xenograft antibodies of the present invention are of the IgM isotype. Even more preferably, the anti-porcine xenograft antibodies of the present invention immunoreact with antigen expressed by pig platelet cells.

In a related embodiment of anti-porcine xenograft antibody of the present invention, the isolated and substantially purified anti-porcine xenograft antibody is further characterized as comprising at least one polypeptide encoded by a nucleic acid sequence which includes a sequence having at least about 90% homology, preferably at least about 95% homology, more preferably at least about 98%

homology, and most preferred about 100% homology to the sequence defined by nucleic acid residues 1 through 291 of SEQ ID NO: 18 or nucleic acid residues 1 through 291 of SEQ ID NO: 20.

In an still another embodiment of the anti-procine xenograft antibody of the present invention, the antibody is characterized as being immunoreactive with antigen expressed by endothelial cells of a pig xenograft, capable of inducing antibody-mediated rejection of the pig xenograft by a recipient animal and comprising at least one polypeptide encoded by a nucleic acid sequence including the sequence defined by nucleic acid residues 1 through 345 of SEQ ID NO: 18 or defined by nucleic acid residues 1 through 357 of SEQ ID NO: 20.

Another selection criterion for presently prefered anti-porcine xenograft antibody is complement-mediated cytotoxicity for pig spleen lymphocytes. Using the flow cytotoxicity assay described herein and pig lymphocytes in place of PAEC, the eleven anti-porcine xenograft monoclonal antibodies were screened for cytotoxicity to pig splenic lymphocytes. All eleven monoclonal antibodies also have the capacity to bind and induce complement-mediated splenic lymphocytes cell death; findings which are characteristic of many preformed human anti-porcine xenograft antibodies. Thus, anti-porcine xenograft antibodies of the present invention preferably are also capable of inducing complement-mediated death of pig spleenic lymphocytes.

Still another selection criterion for presently prefered anti-porcine xenograft antibody is immunoreactivity with proteins having molecular weights similar to those which bind with preformed human anti-porcine xenograft antibodies. Using immunoprecipitation of $^{125}I$ labeled PAEC surface antigens, it was demonstrated that several of the monoclonal antibodies immunoprecipitate proteins of about

the same molecular weight as antigens expressed by PAEC or pig platelet cells and which are recognized by preformed human anti-porcine xenograft antibodies. Preformed human anti-porcine xenograft antibodies bind at least six antigens expressed by pig aortic endothelial cells with molecular weights of 44 kDa, 80 kDa, 115kDa, 125 kDa, 135 kda and 200 kDa by Western blot analysis. Three antigens of similar molecular weights (115kD, 125kD, and 135kD) which are expressed on pig platelet cells are also bound by these preformed human anti-porcine xenograft antibodies.

Four of the eleven anti-porcine xenograft monoclonal antibodies described above and secreted by hybridomas HA73H8, HA72G3, IH21H7, or HA73D7, immunoreact with proteins in the range of 38-44 kDa as demonstrated by immunoprecipitation.

Thus, it is prefered that the anti-porcine xenograft antibodies of the present invention be immunoreactive with at least one antigen expressed by pig endothelial cells or pig platelet cells which are also recognized by preformed human anti-porcine xenograft antibodies. Even more preferably, the anti-porcine xenograft antibodies of the present invention are also capable of blocking preformed human anti-porcine xenograft antibody from binding to PAEC or LCPK1 pig kidney cells. (LCPK1 cells are included in these screening assays because it has been reported that they express the 115 kDa molecule recognized by preformed human anti-porcine IgG antibodies and express αGal terminal residues on surface antigens that are targets of preformed human anti-porcine antibody binding and cytotoxicity. See, Koren, E. et al., "Cytotoxic effects of human preformed anti-gal IgG and complement on cultured pig cells." Second Intern'l Congress on Xenotransplantation, England, September 26-29, 1994; Neethling, F.A., et al., Transplantation 57(6):959-963 1994.) The ability of anti-porcine xenograft

antibodies to block such binding can be screened for in the ELISA format or the flow cytometric assay as described below in the EXAMPLES.

The anti-porcine xenograft antibodies described above were screened for their ability to block preformed human anti-porcine xenograft antibody (as they occur in normal human serum) from binding to PAEC or LCPK1 pig kidney cells. These blocking studies were performed with groups of anti-porcine xenograft monoclonal antibodies in order to screen larger numbers of monoclonal antibodies more efficiently. Each group, comprising four to five individual monoclonal antibodies, was incubated with PAEC or LCPK1 cells. The cells were then incubated with human serum as a source of preformed human anti-porcine xenograft antibodies. Anti-porcine xenograft monoclonal antibodies in Group 1 (HA75D8, IH21H7, HA72G3, HA73D7 and DE101H2) were capable of reproducibly blocking 50-65% of preformed human IgM anti-porcine xenograft antibody binding to LCPK1 cells as indicated by a drop in mean channel shift from 242.5 with human serum alone to 59.9 following preincubation with Group 1 monoclonal antibodies. Group 1 anti-porcine xenograft antibodies were also capable of blocking binding of prefomed human anti-porcine xenograft antibodies to PAEC, indicated by a drop in mean channel shift from 73.6 with human serum alone to 47.2 following preincubation with Group 1 monoclonal antibodies. The rat anti-porcine xenograft monoclonal antibodies in Group 2 (HA73C4, IH27H6, HA71E3, and DE103H6) did not significantly block preformed human IgM anti-porcine xenograft antibody binding to pig cells (mean channel shift of 219.2) in the same experiment but demonstrated an equivalent level of binding to LCPK1 cells as Group 1.

The individual monoclonal antibodies from Group 1 were then each examined for their ability to block preformed

human IgM anti-porcine xenograft antibody binding to pig cells. The results are reported in Table 3.

Table 3. Ability of Rat Anti-Porcine Xenograft Monoclonal Antibodies To Block Binding of Preformed Human Anti-Porcine Xenograft Antibodies to PAEC or LCPK-1 Cells.

Mean Channel Shift (Fluorescence)

| | PAEC | LCPK-1 |
|---|---|---|
| Negative Control | 3.3 | 8.7 |
| Human Serum | 53.1 | 87.7 |
| HA75D8 + Human Serum | 34.0 | 49.8 |
| IH2IH7 + Human Serum | 30.0 | 40.5 |
| HA72G3 | No blocking | No blocking |
| HA73D7 | No blocking | No blocking |
| DE101H2 | No blocking | No blocking |
| | p<.0001 | p<.0001 |

Incubation of HA75D8 monoclonal antibody with PAEC cells or LCPK-1 cells prior to incubation with human serum resulted in a 34.6% and a 37.9% decrease in binding of human serum to PAEC cells and LCPK-1 cells, respectively. Incubation of LCPK1 cells with monoclonal antibody IH2IH7 prior to incubation with human serum resulted in a 46% decrease in binding of human serum to LCPK1 cells. Dilution of monoclonal antibody IH21H7 resulted in a corresponding increase in preformed human IgM anti-porcine xenograft antibody binding to LCPK1 cells, demonstrating specificity of the blocking effect.

B. Anti-Donor Xenograft Antibody Material

In accordance with another embodiment of the present invention there is provided anti-donor xenograft antibody material characterized as immunoreactive with antigen expressed by endothelial cells of a donor xenograft and capable of inhibiting antibody-mediated rejection of the donor xenograft by a recipient animal.

The term "antibody material" in its various grammatical forms is used herein as a collective noun that refers to a population of immunologically active fragments of immunoglobulin molecules, i.e., molecules that contain an antibody combining site. Exemplary antibody materials of the present invention include those portions of immunoglobulin molecules known in the art as Fab, Fab', $F(ab')_2$, and F(v). Fab and $F(ab')_2$ portions of antibodies are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibodies by methods that are well known. See, for example, U.S. Patent No. 4,342,566 to Theofilopolous and Dixon. Fab' antibody portions are also well known and are produced from $F(ab')_2$ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody combining site is that structural portion of the antibody molecule comprised of a heavy and light chain variable and hypervariable regions that specifically binds (immunoreacts with) an antigen.

Thus, the term "anti-donor xenograft antibody material" refers to antibody material that immunoreacts with antigen expressed by endothelial cells of a xenograft and that inhibits antibody-mediated rejection of the xenograft by the recipient animal. In accordance with one embodiment of the

invention, anti-donor xenograft antibody material is selected from the group consisting of Fab, Fab', $F(ab')_2$, F(v) and combinations of thereof. In yet another embodiment of the present invention, anti-donor xenograft antibody material is selected from the group consisting of $F(ab')_2$ and Fab'. In a prefered embodiment of the invention, anti-donor xenograft antibody material is comprises a combination of $F(ab')_2$ and Fab'.

To produce the anti-donor xenograft antibody material of the present invention, anti-donor xenograft antibody can simply be reduced into fragments that retain their ability to immunoreact with antigen expressed by endothelial cells of the xenograft but that are incapable of inducing complement-mediated death of xenograft cells. Alternatively, more sophisticated methods, such as the phage display technique (descibed in greater detail below) can be used to generate anti-donor xenograft antibody material of the present invention.

Screening antibody material for immunoreactivity with antigen expressed by endothelial cells of the xenograft is similar to the method of screening antibodies for the same attribute as described above and in the EXAMPLES, except that an appropriate secondary antibody should be substituted, such as for example enzyme-labeled anti-kappa antibody. The same preference described above regarding the sources of antigen for such screening assays would also apply here.

Likewise, the skilled artisan will appreciate that with the appropriate modifications the same *in vivo* rejection experiments and *in vitro* complement-mediated cytotoxicity assay formats described above for identify antibodies having the ability to induce antibody-mediated rejection of the donor xenograft by a recipient animal can be employed to identify antibody material capable of inhibiting antibody-

mediated rejection of the donor xenograft by a recipient animal.

More specifically, in an *in vitro* format, the inability to induce antibody-mediated, complement-dependent cytotoxicity indicates that that antibody tested has the ability to inhibit antibody-mediated rejection of the donor xenograft by the recipient animal. The source of antigen and complement for the cytotoxicity assays can be as described above for the immunoreactivity assays, or other whole cells from the donor animal can be used as a source of antigen. Preferably, as measured in a flow cytometric cytotoxicity assay, antibody is capable of inhibiting antibody-mediated rejection of a xenograft by a recipient animal when cell death is reduced by at least about 20% as compared to a control, preferably by at about 25% as compared to a control, more preferably by at least about 30% as compared to a control, even more preferably by at least about 35% and most preferably by at least about 40%. One of skill in the art will appreciate that the flow cytometric cytotoxicity assay described in EXAMPLES can easily be adapted for specie combinations of interest.

Alternatively, and more preferably, the ability of anti-donor xenograph antibody material to inhibit antibody-mediated rejection of the xenograft is detected by the ability of the antibody material to block binding of preformed anti-donor xenograph antibodies in the recipient animal serum to antigen expressed by endothelial cells of the xenograft. The ability of anti-porcine xenograft antibodies to block such binding can be screened for in the ELISA format or the flow cytometric assay described in the EXAMPLES below. The ability to inhibit antibody-mediated rejection of the xenograft is detected by the ability of the antibody material to block at least about 20% of the binding detected with normal recipient serum, more preferably at

least about 30% of the binding detected with normal recipient serum, even more preferably at least about 30% of the binding detected with normal recipient serum, and most preferably at least about 50% of the binding detected with normal recipient serum.

The ability of the antibody material to inhibit antibody-mediated rejection of a donor xenograft is identified in the *in vivo* rejections experiments described above when pre-transplant passive transfer of anti-donor xenograft antibody material prolongs the median survival time of the xenograft beyond the median survival time characteristic of the hyperacute rejection of a xenograft by the recipient animal, or more preferably beyond the median survival time of the xenograft characteristic of the accelerated rejection of a xenograft by the recipient animal. When appropriate, post-transplant passive transfer of hyperimmune sera or anti-donor xenograft antibody of the present invention is administered to the transplant recipient in such *in vivo* experiments to generate the characteristic hyperacute rejection of the xenograft, such as is exemplified in the following hamster-to-rat model.

1. Pre-transplant Treatment with Reduced Anti-xenograft Monoclonal Antibody Material Inhibits Hyperacute Rejection

Having demonstrated that the rat anti-donor xenograft monoclonal antibodies of the present invention have specificity for vascular endothelium of hamsters and could induce the hyperacute rejection reaction through activation of the classic complement pathway, rat anti-hamster xenograft antibodies were reduced to antibody material and screened for their ability to inhibit antibody-mediated rejection of the xenograft by rats.

An anti-hamster xenograft monoclonal antibody of the present invention designated HAR-1 above, was reduced to its Fab and $F(ab')_2$ form by protein reduction of the IgM pentamers with 2-mercaptoethanol ("2-ME"). Naive LEW rats were divided into five groups. In group 1, referred to as the "Poly group," naive rats received pre-transplant treatment of a single passive transfer of 0.5 ml rat hyperimmune sera. In group 2, referred to as the "HAR-1 group," the naive rats received pre-transplant treatment of 1.0 ml of HAR-1 (50-100 $\mu$g/protein) in a single passive transfer. In group 3, referred to as "HAR-$1^{red}$/Poly group," the naive rats received pre-transplant treatment of 1.0 ml HAR-1 Fab and $F(ab)_2$ antibody material, followed by passive transfer of 0.5 ml rat hyperimmune sera. Group 4, referred to as "HAR-$1^{red}$/HAR-1 group" received pre-transplant treatment of 1.0 ml HAR-1 Fab and $F(ab)_2$ antibody material, followed by passive transfer of 1.0 ml HAR-1. Group 5, referred to as "9D6/Poly group," received pre-transplant treatment of 1.0 ml 9D6 Fab and $F(ab)_2$ antibody material, followed by passive transfer of 0.5 ml rat hyperimmune sera. (9D6 is an IgM monoclonal antibody generated as described in the EXAMPLES below which does not have binding specificity for hamster endothelium and does not induce antibody-mediated rejection of hamster xenografts by rats.) Each group of rats were then transplanted with hamster cardiac xenografts.

The results shown in Table 2 demonstrate that HAR-1 Fab and HAR-1 $F(ab)_2$ antibody material significantly inhibit the hyperacute rejection of hamster cardiac xenograft ($p<0.01$).

Table 3. Protective Effect of Reduced HAR-1 Antibody Material on Hyperacute Rejection of Hamster Hearts Following Passive Antibody Transfer to Naive Rats

| Group* | N | Survival Time (minutes) | Median Survival Time (minutes) |
|---|---|---|---|
| Poly | 5 | 10, 13, 15, 15, 18 | 15 |
| HAR-1 | 5 | 8, 9, 10, 10, 10 | 10 |
| 9D6/Poly | 5 | 10, 20, 20, 25, 30. | 20 |
| HAR-$1^{red}$/Poly | 5 | 15, 50, 1440, 2580, 1440, 4200, 5400, 6120 | 2580** |
| HAR-$1^{red}$/HAR-1 | 3 | 1440, 4320, 4680 | 4320** |

**Significant at $P<0.01$ in Student's t test when compared with the 9D6/Poly group.

The data indicates that (1) HAR-1 has specificity for the xenoantigen(s) which are primarily responsible for generating antibody-mediated hyperacute rejection and that are also recognized by the antibodies of transplanted rat sera; and (2) the usage of anti-donor xenograft antibody material (e.g., Fab, $F(ab)_2$) can inhibit antibody-mediated xenograft hyperacute rejection.

Thus, in accordance with the present invention there is provided isolated and substantially purified anti-donor xenograft antibody material ("anti-hamster xenograft antibody material") which is characterized as immunoreactive with antigen expressed by endothelial cells of a hamster

xenograft and capable of inhibiting antibody-mediated rejection of the hamster xenograft by a recipient animal. Preferably, the hamster xenograft is heart tissue or the recipient animal is a rat. More preferable the hamster xenograft is heart tissue and the recipient animal is a rat.

In a related embodiment of the anti-hamster xenograft antibody material, the isolated and substantially purified anti-hamster xenograft antibody material is further characterized as comprising at least one polypeptide encoded by a nucleic acid sequence which includes a sequence having at least about 90% homology, preferably at least about 95% homology, more preferably at least about 98% homology, and most preferred about 100% homology to the sequence defined by nucleic acid residues 58 through 351 of SEQ ID NO: 1 or by nucleic acid residues 168 through 440 of SEQ ID NO: 9.

In an still another embodiment of the anti-hamster xenograft antibody material of the present invention, the antibody material is characterized as being immunoreactive with antigen expressed by endothelial cells of a hamster xenograft, capable of inhibiting antibody-mediated rejection of the hamster xenograft by a recipient animal and comprising at least one polypeptide encoded by a nucleic acid sequence including the sequence defined by nucleic acid residues 58 through 420 of SEQ ID NO: 1 or by a nucleic acid sequence including the sequence defined by nucleic acid residues 1 through 354 of SEQ ID NO: 3. Preferably, the anti-hamster xenograft antibody further comprises at least one polypeptide encoded by a nucleic acid sequence including the sequence defined by SEQ ID NO: 5.

In yet another embodiment of the present invention there is provided isolated and substantially purified anti-donor xenograft antibody material ("anti-porcine xenograft antibody material") characterized as being immunoreactive with antigen expressed by endothelial cells of a pig

xenograft and capable of inhibiting antibody-mediated rejection of the pig xenograft by a recipient animal. In a particular embodiment of the invention, the pig xenograft is heart tissue or liver tissue. In another embodiment of the invention the recipient animal is a human.

In a related embodiment of anti-porcine xenograft antibody material of the present invention, the isolated and substantially purified anti-porcine xenograft antibody material is further characterized as comprising at least one polypeptide encoded by a nucleic acid sequence which includes a sequence having at least about 90% homology, preferably at least about 95% homology, more preferably at least about 98% homology, and most preferred about 100% homology to the sequence defined by nucleic acid residues 1 through 291 of SEQ ID NO: 18 or by nucleic acid residues 1 through 291 of SEQ ID NO: 20.

In an still another embodiment of the anti-porcine xenograft antibody material of the present invention, the antibody material is characterized as being immunoreactive with antigen expressed by endothelial cells of a pig xenograft, capable of inhibiting antibody-mediated rejection of the pig xenograft by a recipient animal and comprising at least one polypeptide encoded by a nucleic acid sequence including the sequence defined by nucleic acid residues 58 through 345 of SEQ ID NO: 18 or defined by nucleic acid residues 1 through 357 of SEQ ID NO: 20.

### C. Anti-donor Xenograft Monoclonal Antibodies Show Sequence Homology to One Another and to Germline Sequences

A surprising and unexpected attribute of the anti-donor xenograft antibody material of the present invention, as demonstrated for example by HAR-1 antibody material, is that anti-donor xenograft antibody material could inhibit

antibody-mediated rejection of a xenograft and that polyclonal anti-donor xenograft antibody material was not required. This result suggest that the preformed anti-donor xenograft antibodies responsible for mediating xenograft rejection are polyreactive. Without being bound by any particular theory, it is presently believed that hyperacute rejection of xenografts is mediated by preformed polyreactive anti-donor xenograft antibodies having $V_H$ germline configurations. This belief is substantiated by the nucleic acid sequence homology in heavy chain variable segments utilized in the anti-donor xenograft antibody material sequenced to date and the homology of these sequences to germline sequences of the animal from which the antibody material originates.

Immunoglobulin heavy and light chain cDNA libraries were constructed from mRNA of hybridomas producing rat anti-xenograft monoclonal antibodies. As described in greater detail in the EXAMPLES below, the PCR technique was employed with primers for the proximal part of the constant region of the heavy ($\mu$) or light ($\kappa$ and $\lambda$) chain to synthesize a first strand cDNA from mRNA isolated from the HAR-1-producing the hybridoma and the ID12BF3-producing hybridoma. After the synthesis of the second strand cDNA by standard techniques, a double-stranded synthetic linker of known sequence was ligated to the 5' end of the cDNA to facilitate the amplification of all V, D, and J genes rearranged to the constant region.

The cDNA was amplified by PCR using a second upstream constant region primer and the sense strand of the linker. The amplified product was cloned into the pCR® vector of the TA cloning kit® (Invitrogen, San Diego, CA) to establish the library. Recombinant clones were screened directly from bacterial colonies by PCR and their nucleic acid sequences characterized. This method was found to be more powerful

and practical to use than anchored PCR, which utilizes the addition of a 3' tail to the first strand cDNA to clone sequences with unknown 5' ends.

Due to the limited information available on rat immunoglobulin variable region gene sequences, the boundaries of individual V, D, and J segments, based on consensus-conserved regions, were determined by computer-assisted comparisons with mouse and rat sequences available in the GenBank Database® and by comparison with published germ-line sequences. See, Kabat, E.A., et al. Sequences of proteins of immunological interest. *Department of Health and Human Services*, Anonymous Washington, DC (1991), incorporated herein by reference. The potential introduction of mutations by PCR amplification was compensated for by sequencing and aligning at least 3 individual clones and, whenever possible, by using another DNA polymerase (Ultma DNA polymerase) that has high 3'-5' exonuclease activity that leads to a "proof-reading" activity and suppresses the small misincorporation rate seen with the enzyme Taq DNA polymerase.

The HAR-1 cDNA sequence of the heavy chain variable region is shown in SEQ ID NO. 1 and Figure 1. The ID12BF3 cDNA sequence of the heavy chain variable region is shown in SEQ ID NO. 3. The $V_H$ segments of these two rat anti-hamster xenograft monoclonal antibodies which were generated in separate by fusions are nearly identical to one another. When the sequence of this $V_H$ segment alone was compared to sequences available on GeneBank®, three rat sequences (Accession Nos. RRIGCD25H, RNIGHCAZ, and RNIGHNCS) were identified as having about 91% homology. The classification of rat $V_H$ segments into different families and subfamilies is not yet available, however, using the criterion for membership in human $V_H$ gene families, the four rat $V_H$ gene sequences would belong to the same $V_H$ family. Six mouse

sequences were also found to have about 87% homology to the rat anti-hamster xenograft monoclonal antibody $V_H$ segment.

HAR-1 and ID12BF3 monoclonal antibodies were found to utilize different $J_H$ segments. The cDNA sequence of the HAR-1 $J_H$ segment was found to be 98.2% homologous to the rat germline $J_H1$ gene: identical except for a substitution C for A in the first codon of germline $J_H1$. The cDNA sequence of the ID12BF3 $J_H$ segment was found to be 100% homologous to the rat germline $J_H2$ gene.

Due to possible N region and P element variations during the recombination process, it was not possible to clearly set the boundaries for $D_H$ segment of HAR-1 and ID12BF3, although it is evident that the D segments of these antibodies differ from one another. Nevertheless, a search was also conducted to identify any known sequences with greater that 70% homology to the totality of the cDNA sequence between the end of the $V_H$ segment and the beginning of the $J_H$ segment of HAR-1. No relevant match was found with any of the sequences of the Genebank® database.

The nucleotide and deduced amino acid sequences of the variable region for HAR-1 kappa light chain, were also used to search the GeneBank® database for homologous sequences. The nucleic acid sequence of the HAR-1 $V_\kappa$ segment has about 90% sequence homology to three anti-DNA antibody $V_K$ segments which are all members of the $V_\kappa8$ family defined in mice. A germline counterpart for the HAR-1 $V_\kappa$ segment has not yet been identified, but a search of the Genebank® database has demonstrated that the $J_\kappa$ segment of HAR-1 matches the first 36 nucleotides of the rat germline $J_\kappa2$ (Genebank® Accession No. RNIGKJCA) with 100% identity.

To determine the level of homology between the $V_H$ segment utilized in HAR-1, ID12BF3, and HA75D8F1 and their germline counterpart, genomic liver DNA was amplified and sequenced in accordance with the EXAMPLES below. See also,

Shirwan et al., J. Immunol. 151:5228-5238 (1993) and Shirwan et al., J. Immunol. 150:2295-2304 (1993), incorporated herein by reference. The objective was to establish whether the $V_H$ segments were in a germline configuration or if they were displaying mutations suggesting an antigen-driven affinity maturation. For this purpose, two oligonucleotides (SEQ ID NO. 7 and SEQ ID NO. 8, referred to as RVH1 and RVH2, respectively in Figure 1) that allowed almost complete recovery of the sequence information for the $V_H$ segment of HAR-1 and ID12BF3 were used to amplify genomic liver DNA extracted from a newborn LEW rat. Genomic sequence information was obtained from 3 individual clones. Likewise, two oligonucleotides (5' GGC ACA GAA GTA CAT GGC CG 3' referred to herein as "H7RVH2" which anneals to the framework 3 region of the variable heavy chain and 5' CGT TTA GTT AAT TCA TTA TGC 3' referred to herein as "HA7RVH3" which anneals upstream of the initiation codon of HA75D8F1) that allow almost complete recovery of the sequence information for the $V_H$ segment of HA75D8F1 were used to amplify genomic liver DNA extracted from a newborn LEW rat genomic sequence information was obtained from two individual clones.

Alignment of germline DNA (SEQ ID NO. 9 and referred to herein as $V_H$1.1) and cDNA sequences for the HAR-1 $V_H$ segment is shown in Figure 2. Three differences were found over a total of 376 nucleotides; the $V_H$ segment was 99% homologous to the germline sequence. One transversion C for G led to a replacement of leucine by valine in the eleventh amino acid position of the leader sequence. The two other differences, A for G at position 48 of framework region 1, and T for C at position one of framework region 2, were silent. Alignment of VH1.1 and the cDNA sequence for ID12BF3 also showed about 99% homology to VH1.1, but as a result of different nucleic acid substitutions.

Alignment of germline DNA (referred to herein as VHRAP.1a) and the cDNA sequence for HA75DBF1 $V_H$ segment is shown in Figure 4. Three differences were found over a total of 290 nucleotides; the $V_H$ segment was 98.6% homologous to the germline sequences. All three of these differences occur in the framework regions of the cDNA sequence of HA75D8F1.

The level of the sequence homology, the location of nucleic acid substitutions, and failure of these substitutions to significantly change the deduced amino acid sequence of the $V_H$ segment, provide further evidence that the hyperacute rejection of xenografts is mediated by polyreactive anti-donor xenograft antibodies having $V_H$ germline configurations.

For comparison, the genetic characteristics were also determined for the 9D6 monoclonal antibody. The cDNA sequence of the $V_H$ segment of 9D6 demonstrated a high percentage of identity with three members of the $V_H4$ family as defined in mice. Comparison of heavy chain variable regions of HAR-1 and 9D6 demonstrated an overall homology of 77%. The complementarity determining regions (CDRs) exhibited homologies of 40%, 29%, and 33% for CDR1, CDR2, and CDR3 regions, respectively. The low level of identity in these antigen binding regions is consistent with differences in the ability of these two antibodies to induce hyperacute rejection of hamster hearts.

Finally, cDNA libraries of immunoglobulin $V_H$ genes were constructed from splenic B lymphocytes of newborn rats, naive adult rats, rats which received hamster-xenografts at Day 4 post-transplant, and rats which received hamster-xenografts at Day 21 post-transplant, to establish which germline sequences was utilized for $V_H$ segments *in vivo* by the recipient animals. The precursory frequency of the $V_H1.1$ germline sequence was established by dot hybridization

of immunoglobulin specific gene libraries with primers specific for the $V_H1.1$ gene segment. See, J. Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, p. 9.52-9.55 (1989), incorporated herein by reference. The frequency of $V_H1.1$ gene expression was 1.2% and 1.0% in the newborn and naive adult animals, respectively. The gene frequency was increased to 16% in the recipients of hamster xenografts at Day 4 post-transplant and 10.3% in the recipients at Day 21 post-transplant. Thus, the B cell subset(s) expressing the specific IgM $V_H$ gene utilized in mediating the primary humoral response of the rats to hamster xenografts exist at substantial levels in newborn and adult animals. These B cells undergo rapid clonal expansion and express the $V_H$ gene in a germline configuration after the challenge of rat recipients with hamster xenografts. Sequence analysis of clones from these libraries have demonstrated that the $V_H$ genes used in response to the hamster heart xenografts as well as the porcine xenografts are restricted to a small number of closely-related genes.

The experiments demonstrating the use of Ig genes in a germline configuration for controlling the immune response to xenografts suggests that the B-1a/B-1b B cell pathway is important for the accelerated ("concordant") model of xenograft rejection. This is an unexpected and potentially important observation as this form of antibody production is generally accepted to be responsible for the hyperacute forms of xenograft rejection in more distantly related species combinations. The data presented here suggest that these two patterns of rejection (hamster-to-rat and pig-to-human) share the same Ig gene usage for the control of the humoral immune responses responsible for xenograft rejection. The basic features of the xenograft rejection, despite the involvement of widely divergent species,

represent a rather stereotypical and relatively primitive antibody response to endothelial antigens expressed by the graft. The binding of these antibodies to similar antigens expressed by many different species may be central theme in the pathogenesis of the reaction and may provide the opportunity to specifically target critical steps in the rejection reaction. The ability of a single monoclonal antibody to block the rejection of hamster heart xenografts by rat recipients is clear support for the concept that antibodies and/or their derived fragments will have a role in preventing the xenograft reaction in many species, including the pig-to-human combination.

### D. Nucleic Acids and Polypeptides Encoding Anti-donor Xenograft Antibodies and Antibody Material

The present invention also encompasses isolated and purified polynucleotide molecules encoding antibodies, antibody material, and polypeptide of the present invention. This invention also encompasses polynucleotide molecules characterized by conservative changes in coding regions that do not alter the phenotype of the polypeptide produced therefrom when compared to the nucleic acid molecule described herein.

This invention provides isolated and purified polynucleotides comprising a nucleic acid sequence encoding at least one of the following polypeptides: the polypeptide defined by amino acid residues 1 through 354 of SEQ ID NO: 2; the polypeptide defined by amino acid residues 1 through 402 of SEQ ID NO: 5; the polypeptide defined by amino acid residues 1 through 354 of SEQ ID NO: 3; the polypeptide defined by amino acid residues 1 through 345 of SEQ ID NO: 18; the polypeptide defined by amino acid residues 106 through 151 of SEQ ID NO: 20. The present invention also

provides isolated and purified polypeptides defined by these amino acid sequences.

As used herein, the term "polynucleotide" refers to a contiguous sequence of DNA, RNA, or preferably cDNA. In addition, as used herein, the term "polypeptide" encompasses any naturally occurring allelic variant thereof as well as man-made recombinant forms.

## E. The Phage Display Technique For Generating Anti-Donor Xenograft Antibody Material

As discussed above, the present invention specifically contemplates the use of the phage display technique in combination with PCR amplification of immunoglobulin heavy and light chain libraries as an alternative method of producing and screening for anti-donor xenograft antibody material of the present invention. The phage display technique is particularly useful in producing and screening human anti-donor xenograft antibody material. Although the following discussion is provided in terms of a human as the recipient animal and a pig as the donor animal, the skilled artisan will appreciate that the same technique can be modified for use with other animals and animal combinations.

Family specific primers are employed to generate immunoglobulin heavy chain (consisting of $V_H$ region and part of the $C_{\mu 1}$) and light (kappa) chain ($V_K$, $C_K$) cDNA libraries. By combining these heavy and light chain fragments in a random fashion and inserting them into a phagemid expression vector, the heavy and light chain insert expression product is targeted to the periplasm of *E. coli* for the assembly of heterodimeric Fab molecules. In order to obtain expression of antibody Fab libraries on a phage surface, the nucleotide residue sequence encoding either the heavy or light chains must be operatively linked to the nucleotide residue sequence encoding a filamentous bacteriophage coat protein

membrane anchor. A preferred coat protein for use in this invention in providing a membrane anchor is cp III. In the EXAMPLES described herein methods for operatively linking a nucleotide residue sequence encoding a heavy chain to cp III membrane anchor in a fusion protein of this invention are described.

Preferably a phagemid vector is selected that contains a single cistron consisting of an expression control sequences operatively linked to a periplasmic secretion signal (pelB leader) and a sequence encoding cpIII. The presence of the pelB leader facilitates the secretion of the heavy and light immunoglobulin chains from the bacterial cytoplasm to the periplasmic space where it is cleaved off, while cpIII provides a membrane anchor. The phagemid expression vector suitable for production of the antibody material of the present invention should also carry a selectable resistance marker gene, a phage origin that allows the vector to be replicated as a single stranded DNA and subsequently packaged into phage particles and a bacterial origin of replication that allows the vector to be replicated in a suitable host as double-stranded DNA. A presently prefered phagemid is the SurfZap™ Vector provided in a kit by Statagene, La Jolla, California. Preferably, the pelB leader sequence encodes a first restriction site at its 3' end and the cpIII sequence encodes a second restriction site at its 5' end. These restriction site allow nucleic acid sequences encoding immunoglobulin light and heavy chains to be inserted in the proper orientation as one continuous strand.

Expression of the cistronic message encoding the pelB-$V_K$-$V_H$-cp III fusion sequence leads to the formation of a continuous amino acid seqence that is delivered to the periplasmic space by the pelB leader sequence. The pelB leader is subsequently cleaved and the $V_K$ - $V_H$ chain is

anchored in the membrane by the cp III membrane anchor domain. The heavy chain in the presence of the light chain assembles to form Fab molecules.

With subsequent infection of *E. coli* with a helper phage, as the assembly of the filamentous bacteriophage progresses, the coat protein III is incorporated on the tail of the bacteriophage thereby displaying the Fab on the exterior of the phage particle. Phage particles diplaying the Fab can then be screened for binding to antigen expressed by endothelial cells of a xenograft by the panning method described in the EXAMPLES. Positive phage can be amplified and large quantities of pure Fab produced by co-infection of phagmid and helper phage. The phagemid immunoglobulin insert can also be sequenced either by isolation of the insert or directly from the phagemid.

Thus, in accordance with the present invention there is provided isolated and purified human anti-donor xenograft antibody material that is immunoreactive with antigen expressed by endothelial cells of the donor xenograft and is capable of inhibiting antibody-mediated rejection of the donor xenograft by a human. Preferably the donor xenograft is donated by a pig, and more preferably, the pig xenograft is a pig heart or pig liver.

In a related embodiment of the invention there is provided the human anti-donor xenograft antibody material described above, further comprising at least one polypeptide encoded by a nucleic acid sequence including the sequence defined by nucleic acid residues 1 through 345 of SEQ ID NO: 18 or residues1 through 357 of SEQ ID NO: 20, and at least one human immunoglobulin light chain. Such antibody materials can easily be generated by modification of the [hage display technique described herein to use these heavy chain sequences instead of the sequences obtained through amiplification of the human heavy chain genome.

In yet another embodiment of the present invention there is provided polynucleotides and polypeptides encoding the antibodies and antibody materials of the present invention. Accordingly, this invention provides recombinant anti-donor xenograft antibody material characterized as immunoreactive with antigen expressed by endothelial cells of a donor xenograft and capable of inhibiting antibody-mediated rejection of the donor xenograft by a recipient animal, wherein said antibody material comprises at least one immunoglobulin light chain polypeptide and at least one immunoglobulin heavy chain variable region polypeptide. Preferably the immunoglobulin light chain polypeptide is human or the immunoglobulin heavy chain variable region polypeptide is human. Even more preferably, both polypeptides are human.

In a related embodiment, nucleic acid encoding these recombinant anti-donor xenograft antibody material is provided. Such nucleic acids can be incorporated into vectors, such as for example phagemid vectors including the SurfZAP vector. As used herein the term "vector" refers to a recombinant DNA molecule capable of autonomous replication in a cell and to which a DNA segment, e.g., gene or polynucleotide, can be operatively linked so as to bring about replication of the attached segment. Vectors capable of directing the expression of genes and coding for one or more polypeptides are referred to herein as "expression vectors."

This invention also provides a vector comprising an isolated nucleic acid molecule such as DNA, cDNA or RNA encoding anti-donor xenograft antibody material or the peptide componetns thereof. Examples of additional vectors useful herein are viruses, such as bacteriophages, baculoviruses and retroviruses, cosmids, plasmids, and the like. Nucleic acid molecules are inserted into vector

genomes by methods well known in the art. For example, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules that base pair with each other and which are then joined together with a ligase. Alternatively, synthetic nucleic acid linkers that correspond to a restriction site in the vector DNA, can be ligated to the insert DNA which is then digested with a restriction enzyme that recognizes a particular nucleotide sequence. Additionally, an oligonucleotide containing a termination codon and an appropriate restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such as neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and antisense RNA. Other means are available and can readily be accessed by those of skill in the art.

Cells transformed with vectors of the present invention are also provided, particularly including *E. coli*.

## F. Methods of Inhibiting Antibody-Mediated Xenograft Rejection

The anti-donor xenograft antibody materials and anti-donor antibodies of the present invention can readily be used in the methods of the present invention.

In accordance with the present invention there is provided novel methods of inhibiting antibody-mediated rejection of a xenograft from a donor animal by a recipient

animal which comprises modifying antigen expressed by cells of the xenograft, without causing lysis of the cells, so as to inhibit specific binding of recipient anti-donor xenograft antibody to said antigen. When in unmodified form, these antigens expressed by cells of the xenograft are capable of inducing an antibody-mediated immune response by the recipient animal which, if untreated, results in the rejection of the xenograft. Antigen targeted for modification in accordance with the methods of the present invention are preferably expressed by endothelial cells of the donor xenograft.

One of skill in the art will appreciate that rejection of a xenograft may involve several immunologic components each having varying degrees of importance as rejection of the xenograft proceeds to completion and is dependent upon the combination of species selected for the xenograft transplant. An objective of the present invention is to disrupt the antibody-mediated (i.e., humoral as opposed to cell-mediated) component of the rejection by directly or indirectly modifying antigen presented by the xenograft in such a manner that immunoreactivity (specific binding) between antigen and recipient anti-donor xenograft antibody is reduced or eliminated.

As used herein with regard to the methods of the present invention, the term "recipient anti-donor xenograft antibody" refers to antibody molecules produced by the individual recipient of the xenograft that immunoreact with, i.e., specifically bind, antigen expressed by the cells of the xenograft and induce antibody-mediated rejection of the donor xenograft. Accordingly, such antibodies may be further designated herein by the species of the individual recipient animal (e.g. human) producing the antibodies and/or the specific type of donor animal (e.g. porcine) from which the xenograft originates. Thus, for example,

recipient anti-donor xenograft antibody produced by a human and having specificity for a xenograft from a pig may be refered to herein as human anti-porcine xenograft antibody. Recipient anti-donor xenograft antibody include anti-donor xenograft antibody that naturally occur in the recipient prior to transplant of the xenograft (preforemd antibodies) and anti-donor xenograft antibodies naturally produced by the recipient after transplant of the xenograft.

One of skill in the art will appreciate that many different strategies can be employed to directly or indirectly modify antigen in accordance with the methods of the present invention. In a presently prefered embodiment, modifying antigen expressed by cells of the xenograft comprises contacting non-lytic, anti-donor xenograft antibody material with the antigen for a time, at a temperature, and at a pH suitable to bind the antibody material to the antigen, wherein said anti-donor xenograft antibody material is characterized as immunoreactive with antigen expressed by endothelial cells of the xenograft and capable of inhibiting antibody-mediated rejection of the xenograft by a recipient animal. In a related embodiment, the non-lytic, anti-donor xenograft antibody material is derived from the same species of animal as the recipient animal.

In accordance with the present methods, nonlytic, anti-donor xenograft antibody material may be contacted with antigen prior to transplant, after transplant or both. Thus, antigen expressed by cells of the xenograft may be modified by contacting the antibody material with the antigen by *ex vivo* perfusion of the xenograft with a solution comprising a preservative for the xenograft, such as for example, Viaspir™ (DuPont-Merck Pharmaceuticals, Co.) and the anti-donor xenograft antibody material for a time,

at a temperature, and at a pH suitable to bind the antibody material to the antigen.

The skilled artisan will appreciate that binding of anti-donor xenograft antibody material with antigen expressed by cells of the xenograft can be acheived and optimized by adjusting such reaction parameters as time, temperature and pH. The methods of the present invention are typically performed at or below room temperature at about physiological pH. Because the methods involve the use of proteins, substantially higher temperatures acidity or alkalinity which would substantially modify the tertiary and quaternary structures of the proteins should be avoided. Accordingly, conditions suitable for performing the methods of the present invention generally range from about 1°C to about 37°C, at about physiological pH. The time for performing the methods, of course, will decrease in relation to the increase in temperature at which the methods are performed.

Alternatively or in addition to the *ex vivo* treatment described above, anti-donor xenograft antibody material can be administered to the recipient of the xenogeneic transplant prior to and/or during the actual transplant operation. Administration of the antibody material for this purpose would be carried out along the lines and in amounts generally known in this art. A therapeutically effective amount would be predetermined and calculated to achieve the desired effect, i.e., prolonging the survival time of the xenograft. The required dosage will vary with the particular treatment and with the duration of desired treatment. Since the level of preformed anti-donor xenograft antibody in the serum of a patient can readily be determined by routine clinical analysis, dosages can be taylored to the needs of the individual transplant recipient. Thus, in yet another embodiment of the present

invention, the continued administration of anti-donor xenograft antibody material post-transplant is contemplated, as needed.

In a related embodiment of the present method for inhibiting inhibiting antibody-mediated rejection of a xenograft from a donor animal by a recipient animal, in addition to contacting the antigen with anti-donor xenograft antibody material, said method further comprises administering to said recipient animal a chemical immunosuppressive agent.

When combination treatment (i.e., dual administration of anti-donor xenograft antibody material and chemical immunosuppressive agent) is employed, dosage levels for the anti-donor xenograft antibody material are comparable to levels presented above. Dosage levels for chemical immunosuppressive agent typically fall in the range of about 1 to 1000 milligrams per kilogram of body weight. Ordinarily, 5 to 750 and preferable 10-500 milligrams per kilograms per dose is effective to obtain desired results. Modes of administration as described above are suitable for administration of chemical immunosuppressive agent.

Exemplary chemical immunosuppressive agents contemplated for use in the practice of the present invention are well-known in the art. Suitable immunosuppressive agents include, for example, Cytoxan (cyclophosphamide) azathioprine (AZA), corticosteroids (such as prednisone), OKT3, FK506, mycophenolic acid or the morpholinethylester thereof, 15-deoxyspergualin, rapamycin, mizoribine, misoprostol, anti-interluekin-1 (IL-2) receptor antibodies, anti-lymphocyte globin (ALG), and the like.
co-administer post-transplant

In yet another embodiment of the present invention there is provided a method of inhibiting antibody-mediated rejection of a pig xenograft by a human, which comprises

modifying antigen expressed by endothelial cells of the pig xenograft, without causing lysis of the cells, to inhibit binding of preformed human anti-porcine xenograft antibody to said antigen, wherein said antigen present in unmodified form induces an antibody-mediated immune response in the human. In a related embodiment, antigen expresesed by endothelial cells of the pig xenograft are modified by contacting non-lytic, anti-porcine xenograft antibody material with said antigen for a time, at a temperature, and at a pH suitable to bind the antibody material to the antigen, wherein said anti-porcine xenograft antibody material is characterized as immunoreactive with antigen expressed by endothelial cells of the xenograft and capable of inhibiting antibody-mediated rejection of the xenograft and is preferably characterized as a human antibody material. Anti-donor xenograft antibody material useful in the methods of the present invention can be polyclonal or more preferably monoclonol. Several such antibody materials have been described above.

G. Methods Of Transplanting A Xenograft

In still another embodiment of the present invention there is provided methods for transplanting a xenograft in a patient, said method comprising contacting said xenograft, prior to transplantation,with anti-donor xenograft antibody material for a time, at a temperature, and at a pH suitable to allow said antibody material to immunoreact with antigen expressed by said xenograft, and then transplanting said xenograft. Such methods can further comprise administering a therapeutically effective dose of said anti-donor antibody material and optionally a chemical immunosuppressive agent to said patient post-transplant

H. Therapeutic Compositions

The present invention contemplates therapeutic compositions useful for practicing the therapeutic methods described herein. Therapeutic compositions of the present invention contain a physiologically tolerable carrier together with anti-donor xenograft antibody material, as described herein, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not immunogenic when administered to a mammal or human patient for therapeutic purposes.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art. Typically such compositions are prepared as injectables either as liquid solutions or suspensions; however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents,

pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable nontoxic salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Also illustrative of such acid addition salts are hydrobromide, sulphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, zinc, iron or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

## I. Methods of Isolating Antigen Expressed by Endothelial Cells of the Donor Xenograft

The anti-donor xenograft antibodies and antibody materials of the present invention are particularly well suited to isolate antigen expressed by endothelial cells of a xenograft and which are characterized as inducing antibody-mediated rejection of the xenograft by a recipient animal. Accordingly, the present invention provides methods of isolating such antigen comprising contacting anti-donor xenograft antibody or anti-donor xenograft antibody material with endothelial cell membrane lysate of the xenograft for a time and at a temperature and pH suitable to form an immunecomplex comprising said antibody, then seperating said immune-complex from said non-complexed endothelial cell membrane lysate, and seperating said anti-donor xenograft antibody or antibody material from said antigen. Such antigens include antigen which is characterized as inducing antibody-mediated rejection of the xenograft by humans, particularly those expressed by endothelial cells of porcine xenografts.

Such antigens can readily be isolated by immunoprecipitation with the antibody or the antibody material of the present invention. For example, PAEC can be radiolabeled by growing the cells in the presence of radioactive amino acids or radioactive amoino acid precursors, as described for example in Harlow and Lane, Antibodies: A Laboratory Mannual, Cold Sprong Harbor Laboratories, pp. 430-433 (1989), incorporated herein by reference. Labeled cells can then be lysed as described by Harlow and Lane, pp. 449, *supra*, incorporated herein by reference, and preferably the membrane lysate seperated for immunoprecitation. Antigen can then be immunoprecipitated from the lysate as described by Harlow and Lane, pp. 465, *supra*, incorporated herein by reference. Immune-complex can

then be seperated on two-dimensional SDS-PAGE gels, extracted and seperated by high pressure liquid chromatography.

The invention will now be described in greater detail by reference to the following non-limiting examples.

## V. EXAMPLES

The choice of the hamster-to-rat as the xenogeneic transplant model was based on the consistently observed patterns of rejection for cardiac xenografts among these animals which are accepted as representing both accelerated and hyperacute patterns of xenograft rejection. The pig-to-rat model was also employed since pigs provide a preferred choice for xenogeneic organ transplant to humans because they can be bred easily, their organs are similar in size and function to human organs, and there is a reduce risk of disease transmission to man from the use of pig organs for transplant as compared to non-human primate organs.

All rodent animals brought into the colony are certified virus-free and the colony is monitored regularly for accidental contamination with infectious diseases. The animals are maintained in individual micro-isolator cages, inspected twice daily and fed standard rodent pellet diet and water *ad libitum*. Any procedures that might have produced pain or discomfort to these animals was conducted under fluothane and/or pentobarbital anesthesia. The method of euthanasia is by exsanguination.

The animal facilities at Cedars-Sinai Medical Center are accredited by the American Association for Accreditation of Laboratory Animal Care and the animals included in these studies were handled humanely in accordance with animal

experimental protocols approved by the Institutional Animal Care and Use Committee.

Adult LEW rats (6-8 weeks old) were purchased commercially from Harlan Sprague-Dawley (Indianapolis, IN). Donors for the appropriate xenografts and other lymphoid or endothelial tissues included young Syrian Golden hamsters (Harlan Sprague-Dawley), DBA/2 mice (Jackson Laboratories, Bar Harbour, ME), newborn NZ rabbits (200-250 gm; Irish Farms) and young (220-260 gm) Hartley guinea pigs (Charles River Breeding Laboratories, Wilmington, MA).

Farm pigs used in the routine surgical training of residents in the Department of Surgery at Cedars Sinai Medical Center were used as a source of pig tissue for these experiments.

**Example 1 - Xenograft Transplant**

Intra-abdominal heterotopic ACI rat, hamster, mouse, rabbit and guinea pig cardiac xenograft transplants into rats are performed in accordance with the techniques described in Cramer, D.V. et al., Transplantation, 53: 303-308 (1992), incorporated herein by reference. The donor animals are anesthetized with ketamine (100 mg/kg), xylazine (10 mg/kg), and atropine (0.05 mg/kg) administered intraperitoneally, and then maintained as necessary on methoxyflurane via inhalation. The venae cavae and the pulmonary veins are ligated with 5-0 silk, and the pulmonary artery and aorta are transected 2-3 mm above their origins in the heart. After perfusion of the ventricles and atria with lactated Ringer's solution (containing 200 units/ml of heparin), the heart is placed in a saline bath at 4°C. Recipient animals are anesthetized as described above, a midline incision is made, and the great abdominal vessels are dissected free from the left renal vein to the

bifurcation. The graft is implanted in the abdominal cavity with end-to-side anastomoses of the donor to recipient aortas and of the pulmonary artery to recipient vena cava in a running continuous suture with 10-0 Novafil on a TE-70 needle. Operative times range from 30 to 45 min, with a success rate of approximately 90%. The grafts are evaluated for function by abdominal palpation and all grafts are removed for examination at the termination of the experiment. At removal the hearts are fixed in 10% buffered formalin for 24 hr and then stored for histological processing.

Rejection is considered to have occurred when the xenograft stops functioning, i.e., heart stops beating. Hyperacute rejection is considered to have occurred when the xenograft is rejected within one hour, or more preferably within 10 minutes. Accelerated rejection is considered to have occured when the xenograft has survived for for at least about a

**Example 2 - Histopathology and Antigen Distribution**

Histologic and immunopathologic examinations of the cardiac xenografts and the major organs from normal hamsters to study rat anti-hamster xenograft monoclonal antibody target antigen distribution are performed as described previously in Cramer, D.V., et al. J. Heart Lung Transplant, 11: 458-466 (1992), incorporated herein by reference. Briefly, hamster heart samples are collected immediately following rejection or at 48 hours in those instances in which hyperacute rejection does not occur. After harvesting, the cardiac xenografts are immediately washed with a 0.9% NaCl solution. One portion of the graft is embedded in O.C.T. compound (Tissue-Tek, Miles Inc. Elkhart, IN) and frozen by immersion in an iso-pentane solution

prechilled in dry ice. The remainder of the graft is fixed in 10% buffered formalin and prepared for routine histological examination.

Five micron cryostat sections are cut from frozen tissues and examined with immunofluorescent staining for the deposition of rat immunoglobulins and complement. Tissue sections are incubated with the antibody of interest, followed by isotype specific antibody and an enzyme-labeled secondary antibody, e.g., mouse monoclonal antibody against rat IgM $\mu$ chains (MARM-4, BPS, Inc., Indianapolis, Indiana), goat-anti-rat complement $C_3$ antibody (The Binding Site, Birmingham, England) and FITC-conjugated goat-anti-mouse IgG (Caltag Laboratory, Ontario, Canada) may be used.

To block nonspecific binding, normal hamster serum is added to the secondary antibody in PBS (1:10; v/v). The immunoperoxidase sections are chromonized with 3,3'-Diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical) and counterstained with diluted hematoxylin. Immunofluorescent slides are studied under a Nikon fluorescent microscopy with absorbance at 490 nm.

**Example 3 - Monoclonal Antibody Production**

Rat anti-donor xenograft monoclonal antibodies (hamster or pig donors) are prepared by transplantation of LEW rats with hamster heart xenografts or immunization of LEW rats with isolated PAEC in suspension. Although the following example is discussed in terms of hamster/rat and pig/rat species combinations, the skilled artisan will appreciate that the following method can be modified to generate monoclonal antibodies for other species combinations.

For the production of rat anti-hamster xenograft monoclonal antibodies, LEW rats are transplanted with hamster heart xenografts as described in EXAMPLE 1. The

xenograft is rejected at Day 4 post-transplant and the recipient spleens harvested for cell fusion at rejection.

For the production of rat anti-porcine xenograft monoclonal antibodies, LEW rats are initially immunized by intraperitoneal injection with $10^5$ whole PAEC in PBS followed by a second immunization on day 14 with $10^7$cells in PBS. A third immuniztion of 5 x $10^6$ cells is administered at day 21. At day 24 the rat's spleen is harvested for cell fusion.

The splenic lymphocytes ($10^8$ cells) are mixed with YB2/0 rat myeloma cells (ATCC, Rockville, MD) in serum-free DMEM at a 1:1 ratio, and centrifuged at 500 xg for 5 minutes to pellet the cells. After removing the supernatant, 1 ml of prewarmed (37°C) 50% PEG is added to disrupt the cell pellet. The suspension is swirled for 2 minutes at 37°C, and the mixture diluted with 1 ml of DMEM for 1 minute, followed by the addition of another 1 ml DMEM for one more minute, then 7 ml of DMEM over a course of 2 to 3 minutes. The cells are then centrifuged at 500 xg for 5 min. The cell pellet is gently resuspended in 10 ml of 20% complete DMEM, then diluted to a concentration of 2.5 x $10^6$ cells/ml. Aliquots (0.2 ml) of the cell mixture are placed in individual wells (96 well plates) and incubated at 37°C in 7% $CO_2$.

Hybridoma cells are selected by incubation in HAT medium for a minimum of 2 weeks, beginning on the day after fusion. Supernatants from individual wells are screened for production of IgG and IgM antibodies in an ELISA format as described in EXAMPLE 5 and the ability of the antibodies to immunoreact with antigen expressed by hamster endothelium as described in EXAMPLE 6 or PAEC as described in EXAMPLES 7, below. Clones derived from wells that are positive in the screening process are cloned by limiting dilution in 20% DMEM. Several weeks after the initial cloning, positive

clones are subcloned again by limiting dilution at 0.3 cells/well. The antibody secreting clones are then analyzed by the assays described herein.

**Example 4 - Cell Culture Techniques**

Pig aortic endothelial cells. Pig thoracic aortas are removed using sterile conditions and placed in RPMI/antibiotic medium (RPMI containing penicillin, streptomycin and glutamine (1x antibiotic)). Three 50 ml tubes are filled with RPMI/antibiotic medium, approximately 35 mls are needed to cover the length of the aorta. Harvested aortas are each placed in a petri dish. Using the forceps and scissors the connective tissue (CT) and RBCs are completely removed from the outside of the aorta. RBC contamination will interfere with the culture. Once all the CT is removed, the aorta is placed in the first 50 ml tube containing media and shaken slightly to help remove any RBCs inside the aorta. If the aorta looks clean, it is moved on to tube 2 and then tube 3. Each well of a six-well tissue culture plate (Baxter T4133-2, Corning) that has been coated with 2 ml each of 2% sterile gelatin in 1x PBS (Sigma G-1890) and brought to 37°C, washed with RPMI/antibiotic media (approximately 3 mls) by pipetting media into well, allowed to sit for approximately 15 seconds and discarded into waste. RPMI/antibiotic media (0.5 ml) + 10% FBS is added to each well. The aorta is removed from the third tube and placed in a clean petri dish. The aorta is turned dorsal side up and the aorta cut open longways. A 1.5 cm section of the aorta is gently scraped with surgical blade and the blade is dipped into one well of the 6 well plate. Scraping is repeated in 1.5 cm increments for all six wells. Each well is mixed to help break up the cells. If sheets of cells are seen under a microscope, resuspend more. If no

cells are seen, the aorta is scraped again. The plate is placed in $CO_2$ incubator.

Endothelial cells (PAEC) are passaged when they reach confluence. Experiments are performed on cells between passages 4 and 14, preferably using cells from the earlier passage rather than the latter.

LCPK1 cells. LCPK1 cells (also referred to as minipig kidney cells) can be obtained from the ATCC, Accession No. CRL-1392. Once cells are thawed and rest overnight, the cells are rinsed with serum free media (5 ml for T75 flask, 7 ml for T162) and the media is discarded. Three mililiters 1x Trypsin-Edta (Gibco) are added and the cells incubate at 37°C, 6% $CO_2$ for 3-5 minutes. When 85-95% are floating an equal volume of growth media is added and the cells pelleted at 1500 rpm at room temperature for 5 minutes. Supernatant is discarded and the cells washed in 3-5 ml growth media and pelleted again. Cells are resuspended in 1 ml growth media per flask and incubated at 37°C, 6% $CO_2$. Cells are maintained at 37°C in M199 growth media (Gibco, Grand Island, NY) supplemented with 3% fetal calf serum. (Irvine Scientific) The cells are passaged when they reach 90% confluence and are usually ready for use at passages 5 to 10.

Lymphocytes. Lymphocytes are isolated from the spleens of miniature pigs (Charles River Breeding Laboratory, Wilmington, MA). Under sterile conditions, 15-20 g of pig spleen is passed through stainless steel sieves into 20-40 ml of RPMI containing antibiotics (300 $\mu$/ml penicillin and 300 $\mu$g/ml streptomycin), followed by passage through nylon mesh (Tetko, Inc., Monterey Park, CA). The lymphocytes are isolated by density centrifugation (Histopaque 1077; Sigma Chemical Co., St. Louis, MO), washed 3 times with RPMI, and

then pelleted by centrifugation at 1000 xg for absorption studies.

**Example 5 - Immunoglobulin Isotype ELISA**

The isotype of antibodies presents in the serum of recipient animals, secreted by hybridomas, and binding to tissue or cells the donor, particularly endothelial cells and lymphocytes were characterized and quantified in an ELISA format.

After washing, the cells are incubated with monoclonal antibodies (1:10) and antibody binding to the target cells is detected using affinity-purified mouse anti-rat IgG or IgM conjugated with alkaline phosphatase (Accurate Chemical and Scientific Corporation, Westbury, NY). Absorbance at 405 nanometers is determined after the colorimetric reaction is developed for one hour using 1 mg/ml of p-nitrophenyl phosphate and 100 mM diethanolamine in 0.5 mM $MgCl_2$ (Bio-Rad Laboratories, Richmond, CA).

Hybridoma supernatants are screened for immunoglobulin (IgM) production using ninety-six (96) well flat bottom plates (Corning Costar Corporation, Cambridge, MA) coated with goat αrat IgM (Accurate) antibody (1:2500 of 1 mg/ml stock) in 1M carbonate-bicarbonate buffer (Sigma Chemical, Saint Louis, MO) and incubated overnight at 4°C. The plates are blocked with 5% BSA, washed, and incubated at room temperature for 1 hour with the undiluted (neat) hybridoma supernatants. The plates are then washed and incubated with goat αrat IgM alkaline phosphatase conjugated antibody (1:5000) (Accurate Chemical and Scientific, Westbury, NY) at room temperature for 30 minutes. Absorbances at 405 nanometers are determined after the colorimetric reaction is developed for 30 to 45 minutes using 1 mg/ml of p-

nitrophenyl phosphatase and 100 mM diethanolamine in 0.5 mM $MgCL_2$ (Bio-Rad Laboratories, Richmond, CA).

**Example 6 - Antibody Binding to Hamster Cell**

Since hamster endothelial cells can be difficult to culture, hamster tissue sections is used as the source of antigen to detect immunoreactivity of anti-hamster xenograft antibodies, anti-rat xenograft antibody material and preformed rat anti-hamster xenograft antibodies. Of course, one of skill in the art will appreciate that tissue sections for other donor animals can be substituted in the following assay.

Frozen tissue sections are prepared as described above in EXAMPLE 2 and incubated with the antibody, serum or antibody material of interest, followed by isotype specific antibody and an enzyme-labeled secondary antibody, e.g., mouse monoclonal antibody against rat IgM $\mu$ chain or $\kappa$ chain(MARM-4, BPS, Inc., Indianapolis, Indiana), goat-anti-rat complement $C_3$ antibody (The Binding Site, Birmingham, England) and FITC-conjugated goat-anti-mouse IgG (Caltag Laboratory, Ontario, Canada) may be used.

**Example 7 -ELISA for Immunoreactivity Antigen of Donor Cells**

The binding of preformed anti-donor xenograft antibodies in serum, anti-donor xenograft monoclonal antibodies, or anti-donor xenograft antibody material are measured with this colorimetric assay. One of skill in the art will appreciate that the following assay can be modified to detect immunoreactivity with other types of cells than the specific cells discussed below.

Pig aortic endothelial cells (PAEC) are harvested as described in EXAMPLE 4 and cultured in RPMI with 10% fetal calf serum in 96 well plates (Costar Corp., Cambridge, MA) until confluence. The PAEC are then fixed with 0.1% glutaraldehyde. Non-specific binding is blocked by a one hour incubation in RPMI 1640. Blocking solution is removed and monoclonal antibody supernatant, antibody material, or human serum (1:10 dilution) is incubated for one hour at room temperature. The wells are washed twice with RPMI 1640 and antibody binding is detected by adding affinity-purified anti-donor isotype-specific secondary antibody conjugated with an enzyme label, e.g., goat anti-rat IgG or IgM antibody conjugated with alkaline phosphatase or goat anti-human IgG or IgM antibody conjugated with alkaline phosphatase (both available from Accurate Chemical and Scientific Corporation, Westbury, New York). The secondary antibody is allowed to incubate at room temperature for one hour. Following three washes with RPMI 1640, the colorimetric reaction is developed at room temperature for one hour, using 1 mg/ml of p-nitrophenyl phosphate and 100mM diethanolamine in 0.5M MgCl (Bio-Rad Laboratories, Richmond, CA) Absorbance is read on an automatic micro plate reader at 405nm. A reading of at least two times background or a control, and more preferably at least 2.5 times background or a control is considered positive.

**Example 8 - Flow Cytometry**

The results of the ELISAs to detect immunoreactivity of antibody and antibody material can be compared to similar experiments conducted with the same cells using flow cytometric analysis. Flow cytometric analysis detects surface expressed antigens on cells. The cells of interest ($1x10^6$) are incubated with the antibody of interest

(monoclonal supernatant, serum, antibody material). The cells are washed and incubated with 100 μl of a 1/100 dilution (1 mg/ml initial concentration) of FITC-conjugated affinity-purified anti-donor isotype-specific secondary antibody, e.g., mouse anti-rat IgG or IgM (Serotec, Cambridge, England). Following three washes with PBS at room temperature, fluorescence is read on a FACScan (Becton Dickinson).

**Example 9 - Flow Cytometric Cytotoxicity Assay**

This assay measures antibody-mediated, complement-dependent cytotoxicity and is used to monitor antibody or antibody material cytotoxicity for antigen expression on donor animal cells, such as for example, PAEC or hamster lymphocytes. In order to perform this assay, 5 x $10^5$ donor cells (PAEC or hamster spleen lymphocytes) in RPMI are incubated at room temperature or on ice for 30 minutes with serial two-fold dilutions of the antibody or antibody material of interest (e.g., rat anti-porcine xenograft monoclonal antibody or the rat anti-hamster xenograft monoclonal antibodies) in medium. The cells are washed and rabbit serum (Low Tox, Cedarlane Laboratories) is used as a source of complement. Sixty μl rabbit complement is added to the cells and allowed to incubate for another hour. Propidium iodide is added to the samples, incubated for 5 minutes, and washed with PBS in accordance with Wetzsteon, P.J., et al., A. Hum. Immunol. 35: 93-99 (1992), incorporated herein by reference. The samples are then ready to be read by the FACScan. The uptake of propidium iodide is indicative of cell death. More than about 20% cell death is considered to indicate that the antibody is cytotoxic. Rat anti-hamster serum (1:20 dilution) or rat

anti-pig serum (1:20 dilution) is employed as positive controls and normal hamster or pig serum as negative controls. The cytotoxic titre of the supernatants are expressed as a reciprocal of the last dilution exhibiting more than 20% cytotoxicity.

**Example 10 - Western Blots**

Aortic endothelial cell or lymphocyte membranes are extracted as described earlier by Tuso, P.J., et al., Transplantation 56: 651-655 (1993), incorporated herein by reference. Protein is extracted from the membranes with 0.5% Triton X-100 in 0.15 M NaCl, 1 mM EDTA, 0.062 M Tris, 9.2 mM 6-aminocaproic acid, 1 mM N-ethylmaleimide (NEM), 1 mM PMSF at 4°C for 24 hours. The extract is precipitated with ethanol at -70°C and the protein content determined by dye-binding assay as described by Smith. P.K., et al., Proc. Natl. Acad. Sci. USA 85: 4015 (1988), and incorporated herein by reference.

Membrane proteins (10 $\mu$g) are fractionated on a SDS-PAGE gel using a discontinuous method using 10% separating and 3% stacking gels. The proteins are then transferred to nitrocellulose filter (8 hours at 200 mA). The nitrocellulose filters are then incubated with saturating concentrations of the antibody of interest for 1 hour, followed by an alkaline phosphatase-conjugated mouse anti-rat IgM or IgG (Accurate, dilution 1:2500). The nitrocellulos filters are then developed using alkaline phosphatase substrate kit (Bio-Rad Lab, Richmond, CA).

**Example 11 - Radioimmunoprecipitation**

The molecular weight of the target antigens recognized by rat anti-porcine xenograft monoclonal antibodies are

identified by immunoprecipitation, and compared to the molecular weights of the antigens recognized by preformed anti-porcine xenograft antibodies in human serum that react with PAEC.

Pig aortic endothelial cells (5x $10^6$) cultured as described above are harvested using trypsin-EDTA (Gibco-BRL, Gaithersburg,Maryland), resuspended in fresh RMPI-1640 medium supplemented with 10% FCS and incubated for 30 minutes at 37°C to minimize any proteolytic damage to the relevant antigens. The cells are washed with supplemental PBSx1, resuspended in PBS containing 0.5M sodium phosphate buffer, pH 7.4, and labeled with $^{125}I$ by adding, over a period of 15 minutes, 0.5 mCi $NaI^{125}$, 200 $\mu$l of 20 $\mu$M lactoperoxidase, (Signman,St.Louis, MO) and 250 $\mu$l 0.03% hydrogen peroxide, and incubating at 4°C for 15 minutes.

Radiolabeled cells are washed three times with 15mM NaI in PBS and incubated with monoclonal antibody supernatants for one hour at 4°C. The cells are then lysed with double lysis buffer (1% NP-40 and 0.1% SDS) and antigens are precipitated by incubation of the lysate with CNBR-activated Sepharose 4B beads (Pharmacia, Piscataway, NJ) coupled to mouse anti-rat IgG or IgM monoclonal antibodies (Serotec,Oxford, England). The immunoprecipitated pellet is washed extensively, then denatured in sample buffer (dd $H_2O$ 4 ml, Tris 1 ml, Glycerol 0.8 ml, 10%, 1.6 ml 2-ME and 0.4 ml 0.05% BPB). The eluted material is subjected to electrophoresis on a 10% discontinuous polyacrylamide slab gel and transferred to nitrocellulose membrane. Bands were visualized by exposure of dried nitrocellulose blots to Kodak X-Omat film.

## Example 12 - Construction of $V_H$ and $V_L$ Specific Rat Anti-Donor cDNA Libraries

### a. RNA Isolation

Total RNA is extracted from hybridoma cells using a method described by Chomczynsky, P. and Sacchi, N., Analyt Biochem 162:156 (1987), incorporated herein by reference. Briefly, $10^6$ hybridoma cells are washed twice in PBS and resuspended in 1 ml of a solution containing; GuSCN, 4M; NaCitrate pH 7.0, 1.5 mM; and Sarcosyl 0.5%. After phenol/chloroform:isoamyl-OH extraction, total RNA is precipitated twice in isopropanol at -70°C for one hour periods. The final pellet is resuspended in 50 $\mu$l DEPC (Sigma Chemical, Saint-Louis, MO) treated deionized water and the concentration of the RNA established in a spectrophotometer.

### b. Definition and synthesis of primers

All the oligonucleotides used for PCR amplification and colony hybridization were defined using the PCGene® software. The oliogonucleotides were synthesized using core support facilities at the Cedars-Sinai Research Institute.

### c. Construction of $\mu$ and $\kappa$ cDNA libraries

A linker-mediated polymerase chain reaction (PCR) procedure was used to construct the cDNA libraries. A similar procedure for use with T cell receptors is described at Shirwan, H., et al., J. Immunol. 150: 2295-2304 (1993), and Shirwan, H., et al. J. Immunol. 151: 5228-5238 (1993), incorporated herein by reference. This method employs a double-stranded synthetic linker of known sequence ligated to the 5' end of the double stranded DNA and allows the

amplification and characterization of all unknown V, $D_H$, and J genes rearranged to the constant region.

Heavy chain $\mu$ : The first strand of cDNA is transcribed from 3 $\mu$g of total RNA using a $C_\mu$ primer "RCM1" (SEQ ID NO: 13) and a cDNA synthesis kit according to the manufacturer's instructions (Boerhinger-Mannheim, Indianapolis, IN). After the synthesis of double-stranded cDNA duplex, a double stranded synthetic linker "SAX" (SEQ ID NO: 14) is ligated to the 5' end of the double-stranded cDNA. One $\mu$l T4 DNA ligase (Boehringer Mannheim) is added to 5 $\mu$l cDNA sample, 1 $\mu$l SAX/XAS, 2 $\mu$l $dH_2O$, and 1 $\mu$l $T_4$ buffer and incubated at 16°C for 24 hours. The excess linker is removed by microcentrifugation (Microcon-100, Amicon, Beverly, MA) and the purified cDNA used as a template for PCR amplification.

Amplification is carried out using SAX (SEQ ID NO: 14) and the "RCM3" $C_\mu$ primer (SEQ ID NO: 15) as forward and inverse primers, respectively. RCM3 is located upstream of RCM1 on the constant region. (See, Fig.1) Two microliters of cDNA are used in a 25 $\mu$l reaction that contains: RCM3 (50 ng/$\mu$l), 1 $\mu$l; SAX (50 ng\$\mu$l), 1 $\mu$l; Tris-HCl, 10mM; $MgCl_2$, 1.5mM; KCl, 50mM; dATP, dCTP, dGTP, dTTP, 0.2mM each; and Taq DNA polymerase, 1 unit. The PCR conditions are: denaturation at 94°C for 60 seconds, annealing at 60°C for 60 seconds, and extension a 72°C for 60 seconds; 35 cycles on a DNA Thermal Cycler 480 (Perkin Elmer Cetus, Norwalk, CT). Ten microliters of the reaction are run on a 1.4% agarose gel to check for amplification products and to confirm the absence of contamination as demonstrated by the lack of a signal when water alone is used as the negative control.

One microliter of PCR products is cloned at 12°C for 16 hours into the pCR® vector of the TA cloning kit® (Invitrogen, San Diego, CA) in accordance with the manufacture's instructions. Two microliters of this

reaction (10 ng vector) are used to transform "One-Shot E. Coli" by thermic shock according to the manufacturer's instructions (Invitrogen, San Diego, CA). Two hundred microliters of the transformation reaction are spread on Luria-Bertani petri dishes to which ampicillin, 100 ng/$\mu$l (Sigma Chemical, Saint-Louis, MO) and X-Gal (50 $\mu$l, 20 mg/ml) are added.

Light chain κ: The synthesis of double stranded cDNA from 3 $\mu$g of total RNA extracted from HAR-1 cells is similar to the method described for the $\mu$ chain. The synthesis of the first strand, however, uses the oligonucleotide poly$(dT)_{15}$ from the cDNA synthesis kit. A first round of amplification is carried out using SAX (SEQ ID NO 14) and a $C_\kappa$, primer referred to as "RCK1" (SEQ ID NO 16) as forward and inverse primers, respectively. A "proof-reading" DNA polymerase, *Ultma* DNA polymerase (Perkin Elmer) is used to amplify 4 $\mu$l of cDNA in a 100$\mu$l reaction that contained: 10X Ultma Buffer, 1X; $MgCl_2$ 25mM, 1.5mM; dATP, dCTP, dGTP, dTTP, 40$\mu$M each; SAX (50 ng/$\mu$l), 4$\mu$l; RCK1 (50 ng/$\mu$l), 4$\mu$l; Ultma AND polymerase, 0.5 unit. The PCR conditions are: denaturation at 94°C for 45 seconds, annealing at 55°C for 1 second, and extension at 72°C for 60 seconds; 30 cycles on a DNA Thermal Cycler 480 (Perkin Elmer Cetus, Norwalk, CT). The PCR products are fractionated on a 1.2% low melting point agarose gel (Gibco BRL, Gaithersburg, MD) and the cDNA fragments of 492 to 1107 bp purified by phenol/chloroform extraction-ethanol precipitation, resuspended in 15 $\mu$l Tris-EDTA and subjected to another round of PCR amplification.

One microliter of this solution is reamplified using SAX as forward primer and a $C_\kappa$ primer referred to a RCK3 (SEQ ID NO 17) as a reverse primer. RCK3 is located upstream of RCK1 on $C_\kappa$. This second "nested" amplification uses the enzyme Taq as DNA polymerase so as to generate PCR

products with unpaired deoxyadenosine on their 3' ends, a condition necessary for efficient cloning into the pCR® vector. The PCR conditions are as above for Taq, except that the number of cycles is limited to 25. One microliter of PCR products are cloned in the pCR® vector, in accordance with the manufacturer's direction, and *E. coli* bacteria transformed as described above. Two hundred microliters of the transformation reaction are spread on Luria-Bertani petri dishes to which ampicillin, 100 ng/μl (Sigma Chemical, Saint-Louis, MO) and X-Gal (50 μl, 20 mg/ml) are added.

**Example 13 - Screening of μ and κ cDNA Libraries**

Recombinant colonies are first identified by color screening based on the demonstration of the loss of α-complementation. White colonies are subsequently screened by PCR using SAX/RCM3 and SAX/RCK3 as primers for the μ and κ library, respectively. Briefly, each individual colony is harvested with a sterile tooth-pick, resuspended in 100 μl sterile double distilled water, and incubated at room temperature for 30 minutes. Each tube is vortexed briefly and 4 μl used as a template in a 10 μl reaction that includes: SAX (50 ng/μl), 0.8 μl; anti-sense primer (50 ng/μl), 0.8 μl; Tris-HCl, 10mM; $MgCl_2$, 1.5mM; KCl, 50mM; dATP, dCTP, dGTP, dTTP, 0.2mM each; and 1 unit of *Taq* DNA polymerase. The PCR conditions are: denaturation at 94°C for 20 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds; 28 cycles on a GeneAmp PCR system 9600 (Perkin Elmer Cetus, Norwalk, CT). Colonies of interest are identified by the demonstration of an insert of the expected size (~ 600 bp for SAX/RCM3 and 460 bp for SAX/RCK3). Within each library, a minimum of 4 positive colonies are used to obtain cDNA sequence information.

**Example 14 - Plasmid DNA Extraction and Sequencing**

Each positive colony was incubated at 37°C for 18 hours in culture medium containing ampicillin (100 ng/$\mu$l). Plasmid DNA is extracted by alkaline lysis according to Sambrook, J., et al., Molecular Cloning, A Laboratory Manual Cold Spring Harbor Laboratory Press (1989) and sequenced on both strands with the Sanger dideoxynucleotide chain termination method as used in the Sequenase Plasmid Sequencing kit (United States Biochemicals, Cleveland, OH).

**Example 15 - Extraction and Amplification of Germline $V_H$ DNA from Rat Liver**

LEW liver is harvested and digested for 16 hours at 55°C in a solution of proteinase K, 10 mg/ml; Tris, 50 mM; EDTA, 100 mM; NaCl, 100mM and SDS 1%. After phenol/chloroform extraction the DNA is precipitated with ethanol. Ten nanograms of DNA are amplified with Ultma DNA polymerase using the parameters already described for that enzyme. Amplification primers were chosen based on the sequence information for $V_H$ segment of HAR-1(Figure 1). The sense primer referred to here as "RVH1" (SEQ ID NO 7)is designed to anneal immediately before the initiation codon. The anti-sense primer, referred to here as "RVH2" (SEQ ID NO 8), is designed to anneal on framework region 3, downstream of the first two complementarity determining regions. The PCR conditions are as already described for the Ultma enzyme.

The amplification products are treated with Taq DNA polymerase so as to add unpaired deoxyadenosine to the 3' end of the molecules and to allow for subsequent cloning in the pCR® vector. For that purpose, amplification products generated with Ultma are fractionated by electrophoresis on a 1.4% LMP agarose gel, purified by phenol/chloroform,

precipitated in ethanol and resuspended in 20 μl Tris-EDTA. One unit of *Taq* DNA polymerase and 4 μl 1mM dATP are added and the reaction carried out at 72°C for 10 minutes. The excess of *Taq* is removed by phenol/chloroform, the reaction products precipitated by ethanol, and the volume brought back to 20 μl. Two microliters of treated cDNA are cloned in the pCR® vector and *E.coli* transformed as already described. Recombinant colonies are searched for using PCR amplification with RVH1/RVH2. Four separate clones that demonstrate an insert of the expected size (~460 bp) are selected and both strands sequenced.

**Example 16 - Anti-donor Xenograft Antibody Material By Phage Display Technique**

The following method of making anti-donor xenogen antibody material is described in term of a the generation and screening of a human IgM heavy chain, human IgK light chain Fab library displayed through the use of filimentous phage encoded by the SurfZAP™ Vector (Stratagene, La Jolla, CA) and the SurfZAP™ Vector Kit, both available from Stratagene (La Jolla, CA). Where additional detailed is desired, reference should be made to the manufacturer's directions provided with vector and kit, and incorporated herein by reference. One of skill in the art will appreciate that the method can be modified to accomodate the production of other immunoglobulin Fab libraries of the present invention and utilizing other vectors.

**a. Isolation of Human Lymphocytes**

Peripheral blood or perferably splenic lymphocytes are isolated from a human transplant patient who has recieved a porcine xenograft. For example, biopsy tissue is minced and then incubated at 37°C for 30 minutes in sterile culture

medium (RPMI 1640, 10% FCS, and antibiotics) with 20 μg/ml collagenase, 20 μg/ml hyaluronidase, and 0.1% DNase. Tissue is then titrated through an 18 gauge needle until a cloudy suspension is achieved. After washing, the resultant single cell suspension is centrifuged on a Ficoll-Hypaque gradient to obtain mononuclear cells. Peripheral blood mononuclear cells ("PBMC") are isolated directly by Ficoll-Hypaque fractionation.

**b. Confirmation of IgM Isotype and PAEC Binding**

Isolated lymphocytes are cultured at 37°C in a humidified atmosphere of 5% $CO_2$:95% air for 12 days at a concentration of 2 x $10^6$ cells/ml in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum and antibiotics.

Supernatant is analyzed for concentrations of total serum IgM by a standard ELISA method. Briefly, wells of microtiter plates (Costar, Pleasanton, CA) are coated overnight (4°C) with goat anti-human IgM (Accurate Chemical and Scientific Corp., Westbury, NY) diluted in carbonate-bicarbonate buffer, pH 9.6 (Sigma, St. Louis, MO). The plates were rinsed three times for 15 minutes with PBS + 0.5% Tween-20 and incubated for 1 hour at 4°C with serial dilutions for each serum sample, assayed in triplicate, then stained for one hour at 4°C with goat anti-human IgM-alkaline phosphatase. (Accurate Chemical and Scientific Corp., Westbury, NY) Absorbance of 405 nm was determined after the colorimetric reaction was developed using 1 mg/ml of p-nitrophenyl phosphate and 10 mM diethanolimine in 0.5 mM $MgCl_2$ (Bio-Rad Laboratories, Richmond, CA) for 1 hour.

Supernatant are also analyzed for binding to PAEC cells as described above.

c. Library Construction

$V_\mu$ and $V_\kappa$ Library Generation for Phage Display Human Anti-Porcine Xenograft Antibody Library. The nucleotide sequences encoding the immunoglobulin protein CDRs are highly variable. However, there are several regions of conserved sequences in nucleotide sequences encoding human immunoglobulins that flank the V region domains of either the light or heavy chain, for instance and that contain substantially conserved nucleotide sequences, i.e., sequences that will hybridize to the same primer sequence.

Polynucleotide synthesis (amplification) primers that hybridize to the conserved sequences and incorporate restriction sites into the DNA homolog produced that are therefore suitable for operatively linking the synthesized DNA fragments to a vector are employed. More specifically, the primers are designed so that the resulting DNA homologs produced can be inserted into an expression vector in reading frame with the upstream translatable DNA sequence at the region of the vector containing the first restriction site. Alternatively, linkers containing the desired restriction site (e.g., SEQ ID NO: 33) can be blunt end ligated to the end of the Ig DNA fragment so that the resulting DNA homologs produced can be inserted into an expression vector in reading frame with the upstream translatable DNA sequence at the region of the vector containing the first restriction site. Amplification with the primers described herein is performed on cDNA templates produced from mRNA isolated from lymphocytes isolated as described above.

$V_H$ Primers. For amplification of the $V_H$ region, primers are designed to introduce cohesive termini compatible with directional ligation into one of the two unique restriction sites (NotI or SpeI) of the SurfZAP vector. In all cases,

the 5' primers should be chosen to be complimentary to the first strand cDNA in the conserved and N-terminus region (anti-sense strand). Listed in SEQUENCE ID Nos: 26 through 31 are exemplary Ig family specific heavy chain Ig primers which can be engineered to incorporate the desired restriction site or used with restriction site encoding linkers.

Additional $V_H$ amplification primers including the unique 3' primer are designed to be complimentary to a portion of the first constant region domain of the $\mu$1 heavy chain mRNA (SEQUENCE ID No: 32). These primers will produce DNA homologs containing polynucleotides coding for amino acids from the $V_H$ region and the first constant region domains of the heavy chain. These DNA homologs can therefore be used to produce Fab fragments rather than $F_V$.

Additional unique 3' primers designed to be hybridized to similar regions of another class of immunoglobulin heavy chain such as IgG, IgE and IgA are contemplated. Other 3' primers that hybridize to a specific region of a specific class of $CH_1$ constant region and are adapted for transferring the $V_H$ region amplified using this primer to an expression vector capable of expressing those $V_H$ region with a different class of heavy or light chain constant regions are also contemplated.

Either the $V_H$ or the $V_C$ should contain a third restriction, unique from either the first or second restrictions site, to provide a means for directional ligating heavy and light chain sequences together for subesequent insert into the phagemeid vector.

Amplification is performed in six separate reactions, each containing one of the 5' family specific (VH1 - VH6) primers and a 3' primer. The 5' primers preferably incorporate a Not I site and the 3' primers preferably incorporate a FSI 1 restriction site, for the insertion of

the $V_H$ DNA homolog into the phagemid expression vector and ligation to the Ig kappa light chain sequence, respectively.

$V_K$ Primers. The nucleotide sequences encoding the $V_K$ CDRs are highly variable. However, there are several regions of conserved sequences that flank the $V_K$ CDR domains including the $J_K$, $V_K$ framework regions and $V_K$ leader/promoter. Therefore, amplification primers are constructed that hybridize to the conserved sequences and incorporate restriction sites that allow directional ligation of the amplified fragment to the heavy chain fragment.

For amplification of the $V_K$ CDR domains, the 5' primers are designed to be complimentary to the first strand cDNA in the conserved N-terminus region. These primers preferably introduce a FSI 1 restriction endonuclease site to allow the $V_K$ DNA homolog to be ligated to the $V_H$ homolog. The 3' $V_K$ amplification primer is designed to hybridize to the constant region of the kappa mRNA and to introduce the Spe I restriction endonuclease site required to insert the $V_K$ DNA homolog into the SurfZAP vector. These primers allow a DNA homolog to be produced containing polynucleotide sequences coding for constant region amino acids of the kappa chain. These primers make it possible to produce a Fab fragment rather than a Fv. Amplification with these primers is performed in separate reactions, each containing one of the family specfic 5' primers and one of the 3' primers.

Amplification primers designed to amplify human light chain variable regions of the lambda isotype are also contemplated.

$V_H$ and $V_K$ Library Construction. Total RNA is extracted from $1.15 \times 10^7$ lymphocytes using standard guanadinium isothiocynate extraction protocols. See, for example,

Chomcynski, P. and Saochi, N., Anal. Biochem. 162:156-159 (1987), incorporated herein by reference.

In preparation for PCR amplification, the mRNA, prepared above, is used as a template for cDNA synthesis by a primer extension reaction. Thus, 10 μg RNA is reverse transcribed to single-stranded cDNA using 1 μg oligo-dT primer with 10 mM dithiothreitol, RNasin™ (a protein RNAse inhibitor of Promega Corporation, Madison, WI), 25 mM each dATP, dCTP, dGTP, dTTP, 1x reverse transcriptase buffer (Bethesda Research Laboratories, Bethesda, MD), and 2μl (two hundred units) reverse transcriptase (Bethesda Research Laboratories,™ Bethesda, MD) in 50 μl volume for 10 minutes at room temperature followed by 50 minutes at 42°C. Following a 5 minute 90°C heat kill and 10 minutes on ice, the reaction was treated with 1 μl (one unit) RNase H (Bethesda Research Laboratories) for 20 minutes at 37°C.

The cDNA generated above is amplified using the polymerase chain reaction ("PCR") method. Family specific variable region and isotype specific constant region primers as described above are used to create heavy chain IgM $V_H1$-$V_H6$ family-specific and light chain $V_K1$-$V_K3$ family-specific libraries.

PCR amplification is performed in a 50 μl reaction containing the products of the reverse transcription reaction (about 100 μg of the cDNA/RNA hybrid), 50 ng of 5' $V_H$ primer, 50 ng of the 3' primer, 500mM of the mixture of dNTP's, 5 mM KCl, 100 mM Tris-HCl buffer at pH 8.3, and .25 units of Taq DNA polymerase (Boehringer Mannheim, Indianopolis, Indiana). The reaction mixture is subjected to 30 cycles of amplification using a DNA Thermal Cycler 480 (Perkin Elmer Cetus, Norwalk, CT) Each amplification cycle included denaturing of cDNA at 94°C for 15 minutes, followed by annealing of primers at 52°C for 50 minutes, and

amplification at 72°C for 90 minutes. This is followed by a 10 minute extension at 72°C.

After verifying by agarose gel electrophoresis that all amplifications are successful and that similar yields are achieved, heavy chain and light chain libraries are separately pooled and gel purified on 0.8% Seaplaque GTG Agarose (FMC, Rockland, ME) according to the manufacturer's directions.

Equal portions of the products from each light chain primer extension reaction and each heavy chain primer extension reaction is mixed to create randomization of subsequent $V_H$ and $V_K$ ligation. The mixed products are digested with FPI 1 restriction endonuclease. (All restriction enzymes are available from Boehringer-Mannheim, Indianapolis, IN.) Digested products are again gel purified as described above, and the region of the gel containing DNA fragments of appropraite size are excised, electroeluted into a dialysis membrane and ethanol precipitated. The resulting DNA fragments are again mixed to create randomization and liogated to one another, and gel purified as described above. Finally, ligated DNA is double-digested with Not 1 and Spe 1 which will represent a repertoire of polypeptide genes having cohesive termini adapted for directional ligation into the to create an insert that can be directionally ligated into the SurfZAP vector.

The SurfZAP vector is prepared in accordance with the manufacturer's direction for inserting the $V_H$-$V_K$ sequence.

Transformation of Host with Ig Library. *Escherichia coli* provided in the SurfZAP Cloning Kit (Stratagene, La Jolla, CA) are transformed with the SurfZAP vector containing the Ig library in accordance with the manufacturer's directions. Transformants are selected by antibioic resistance and enriched by growth in liquid cultures.

d. Panning

Panning is performed to select for phage displayed Fab that bind PAEC. PAEC cells are grown in in RPMI 1640 growth medium supplemented with 10% FBS and antibiotics to approximately 4 million cells, preferably at a low passage (<2), in a T75 tissue culture flask. The cells are rinsed 5-10 times with PBS to remove all medium. Non-specific binding is blocked with PBS supplemented with 3% BSA for one hour at 37°C. The cells are aspirated to remove all blocking agent and about $10^{11}$ phage in SM broth are added to the cells and gently rocked at 37°C for about 2-3 hours. Cells are washed about 10 times with PBS supplemented with .5% Tween 20 to remove unbound phage.

Phage which bind the cells are eluted by incubating cells with 2 ml of .1M HCl at pH 2.2 with BSA to a concentration 1mg/ml (w/v) for about 10 minutes at room temperatire while gently rocking the flask. The reaction is neutrilized with 2 M Tris base. The number of phage eluted is monitored by CFU.

Eluted phage are amplified by reinfecting 2 ml *E. coli* XL-Blue in growth medium supplemented with tetracycline with 50 μl eluted phage. Ten ml SB with carbenicillin is added to select for phagemids. Panning repeated until there was at least 100 fold enrichment. For enrichment quantitation, aliquots of the original library are re-panned in parallel with each cycle of enrichment to control for daily fluctuations in phage recovery. Enrichment is calculated by ratio of phage on vs. off and compared to the unenriched library run on the same day. Preferably, each round of panning is conducted against PAEC from the same individual and PAEC from another individual as a control.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Cedars-Sinai Medical Center
(B) STREET: 8700 Beverly Boulevard
(C) CITY: Los Angeles
(D) STATE: California
(E) COUNTRY: US
(F) POSTAL CODE (ZIP): 90048-1863
(G) TELEPHONE: (310) 855-5284
(H) TELEFAX: (310) 967-0101

(ii) TITLE OF INVENTION: COMPOSITIONS AND METHODS FOR INHIBITING XENOGRAFT REJECTION

(iii) NUMBER OF SEQUENCES: 33

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/440,621
(B) FILING DATE: 15-MAY-1995

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 420 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: LEW RAT
(F) TISSUE TYPE: Spleen, hyperimmunized
(G) CELL TYPE: Splenic lymphocyte

(vii) IMMEDIATE SOURCE:
(B) CLONE: HAR-1

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..420
(D) OTHER INFORMATION: /product= "Immunoglobulin Variable

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                Region"
                /standard_name= "Ig Heavy Chain Variable Region"
                /label= VH-Region
                /note= "Variable Region of HAR-1 Heavy Chain"

(ix) FEATURE:
      (A) NAME/KEY: misc_RNA
      (B) LOCATION: 1..57
      (D) OTHER INFORMATION: /standard_name= "Leader"
            /label= Leader

(ix) FEATURE:
      (A) NAME/KEY: misc_RNA
      (B) LOCATION: 58..351
      (D) OTHER INFORMATION: /standard_name= "Ig Heavy Chain
            Variable Segment"
            /label= VH-Segment
            /note= "Variable Segment of HAR-1 Heavy Chain
            Variable Region"

(ix) FEATURE:
      (A) NAME/KEY: misc_RNA
      (B) LOCATION: 352..366
      (D) OTHER INFORMATION: /standard_name= "Ig Heavy Chain
            Diversity Segment"
            /label= D-Segment
            /note= "Diversity Segment of HAR-1 Heavy Chain
            Variable Region"

(ix) FEATURE:
      (A) NAME/KEY: misc_RNA
      (B) LOCATION: 367..420
      (D) OTHER INFORMATION: /product= "Immunoglobulin Joining
            Region"
            /standard_name= "Ig Heavy Chain Joining Segment"
            /label= JH-Segment
            /note= "Joining Segment of HAR-1 Heavy Chain
            Variable Region"

(ix) FEATURE:
      (A) NAME/KEY: misc_RNA
      (B) LOCATION: 58..147
      (D) OTHER INFORMATION: /standard_name= "Framework Region
            1"
            /label= FR-1
            /note= "Framework Region 1 of HAR-1 Heavy Chain
            Variable Region"

(ix) FEATURE:
      (A) NAME/KEY: misc_RNA
      (B) LOCATION: 148..162
      (D) OTHER INFORMATION: /standard_name= "CDR-1"
            /label= CDR-1
            /note= "Complimentarity Determining Region 1 of
            HAR-1 Heavy Chain Variable Region"
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(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 163..204
(D) OTHER INFORMATION: /standard_name= "Framework Region 2"
/label= FR-2
/note= "Framework Region 2 of HAR-1 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 205..255
(D) OTHER INFORMATION: /standard_name= "CDR-2"
/label= CDR-2
/note= "Complimentarity Determining Region 2 of HAR-1 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 256..351
(D) OTHER INFORMATION: /standard_name= "Framework Region 3"
/label= FR-3
/note= "Framework Region 3 of HAR-1 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 352..387
(D) OTHER INFORMATION: /standard_name= "CDR-3"
/label= CDR-3
/note= "Complimentarity Determining Region of HAR-1 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 388..420
(D) OTHER INFORMATION: /standard_name= "Framework Region 4"
/label= FR-4
/note= "Framework Region 4 of HAR-1 Variable Heavy Chain Region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG GAC ATC AGG CTC AGC TTG GCT TTC CTT CTC CTT TTC ATA AAA GGT        48
Met Asp Ile Arg Leu Ser Leu Ala Phe Leu Leu Leu Phe Ile Lys Gly
 1               5                  10                  15

GTC CAG TGT GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC TTA GTG CAG        96
Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln
            20                  25                  30

CCT GGA AGA TCC CTG AAA CTC TCC TGT GCA GCC TCA GGA TTC ACT TTC       144
```

```
Pro Gly Arg Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
          35                  40                  45

AGT AAC TAT GGC ATG GCT TGG GTC CGC CAG GCT CCA ACG AAG GGT CTG        192
Ser Asn Tyr Gly Met Ala Trp Val Arg Gln Ala Pro Thr Lys Gly Leu
     50                  55                  60

GAG TGG GTC GCA TCC ATT AGT ACT GGT GGT GGT AAC ACT TAC TAT CGA        240
Glu Trp Val Ala Ser Ile Ser Thr Gly Gly Gly Asn Thr Tyr Tyr Arg
 65                  70                  75                  80

GAC TCC GTG AAG GGC CGA TTC ACT ATC TCC AGA GAT AAT GCA AAA AAC        288
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
                 85                  90                  95

ACC CTA TAC CTG CAA ATG GAC AGT CTG AGG TCT GAG GAC ACG GCC ACT        336
Thr Leu Tyr Leu Gln Met Asp Ser Leu Arg Ser Glu Asp Thr Ala Thr
            100                 105                 110

TAT TAC TGT GCA AGA CAT CGC GGG TAT AAC TCC TAC TGG TAC TTT GAC        384
Tyr Tyr Cys Ala Arg His Arg Gly Tyr Asn Ser Tyr Trp Tyr Phe Asp
        115                 120                 125

TTC TGG GGC CCA GGA ACC ATG GTC ACC GTG TCC TCA                        420
Phe Trp Gly Pro Gly Thr Met Val Thr Val Ser Ser
    130                 135                 140
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Asp Ile Arg Leu Ser Leu Ala Phe Leu Leu Leu Phe Ile Lys Gly
  1               5                  10                  15

Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln
            20                  25                  30

Pro Gly Arg Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
          35                  40                  45

Ser Asn Tyr Gly Met Ala Trp Val Arg Gln Ala Pro Thr Lys Gly Leu
     50                  55                  60

Glu Trp Val Ala Ser Ile Ser Thr Gly Gly Gly Asn Thr Tyr Tyr Arg
 65                  70                  75                  80

Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
```

```
                85                  90                  95

Thr Leu Tyr Leu Gln Met Asp Ser Leu Arg Ser Glu Asp Thr Ala Thr
            100                 105                 110

Tyr Tyr Cys Ala Arg His Arg Gly Tyr Asn Ser Tyr Trp Tyr Phe Asp
        115                 120                 125

Phe Trp Gly Pro Gly Thr Met Val Thr Val Ser Ser
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 354 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: LEW RAT
(F) TISSUE TYPE: Spleen, hyperimmunized
(G) CELL TYPE: Splenic lymphocyte

(vii) IMMEDIATE SOURCE:
(B) CLONE: ID12BF3

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..354
(D) OTHER INFORMATION: /product= "Immunoglobulin Variable Region"
/standard_name= "Ig Heavy Chain Variable Region"
/label= VH-Region
/note= "Variable Region of ID12BF3 Heavy Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..294
(D) OTHER INFORMATION: /standard_name= "Heavy Chain Variable Segment"
/label= VH-Segment
/note= "Variable Segment of ID12BF3 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 295..308
(D) OTHER INFORMATION: /standard_name= "Ig Heavy Chain Diversity Segment"
/label= D-Segment

/note= "Diversity Segment of ID12BF3 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 309..354
(D) OTHER INFORMATION: /product= "Immunoglobulin Joining Region"
/standard_name= "Ig Heavy Chain Joining Segment"
/label= JH-Segment
/note= "Joining Segment of ID12BF3 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..90
(D) OTHER INFORMATION: /standard_name= "Framework Region 1"
/label= FR-1
/note= "Framework Region 1 of ID12BF3 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 91..105
(D) OTHER INFORMATION: /standard_name= "CDR-1"
/label= CDR-1
/note= "Complimentarity Determining Region 1 of ID12BF3 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 106..147
(D) OTHER INFORMATION: /standard_name= "Framework Region 2"
/label= FR-2
/note= "Framework Region 2 of ID12BF3 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 148..198
(D) OTHER INFORMATION: /standard_name= "CDR-2"
/label= CDR-2
/note= "Complimentarity Determining Region 2 of ID12BF3 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 199..294
(D) OTHER INFORMATION: /standard_name= "Framework Region 3"
/label= FR-3
/note= "Framework Region 3 of ID12BF3 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 295..321
(D) OTHER INFORMATION: /standard_name= "CDR-3"
/label= CDR-3
/note= "Complimentarity Determining Region 3 of ID12BF3 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 322..354
(D) OTHER INFORMATION: /standard_name= "Framework Region 4"
/label= FR-4
/note= "Framework Region 4 of ID12BF3 Heavy Chain Variable Region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg
 1               5                  10                  15

TCC CTG AAA CTC TCC TGT GCA GCC TCA GGA TTC ACT TTC AGT AAC TAT        96
Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
            20                  25                  30

GGC ATG GCT TGG GTC CGC CAG GCT CCA ACG AAG GGT CTG GAG TGG GTC       144
Gly Met Ala Trp Val Arg Gln Ala Pro Thr Lys Gly Leu Glu Trp Val
        35                  40                  45

GCA TCC ATT AGT ACT GGT GGT GGT AAC ACT TAC TAT CGA GAC TCC GTG       192
Ala Ser Ile Ser Thr Gly Gly Gly Asn Thr Tyr Tyr Arg Asp Ser Val
    50                  55                  60

AAG GGC CGA TTC ACT ATC TCC AGA GAT AAT GCA AAA AAC ACC CTA TAC       240
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65                  70                  75                  80

CTG CAA ATG GAC AGT CTG AGG TCT GAG GAC ACG GCC ACT TAT TAC TGT       288
Leu Gln Met Asp Ser Leu Arg Ser Glu Asp Thr Ala Thr Tyr Tyr Cys
                85                  90                  95

GCA AGA CCT TCC TAT AGC AGC TAC TTT GAT TAC TGG GGC CAA GGA GTC       336
Ala Arg Pro Ser Tyr Ser Ser Tyr Phe Asp Tyr Trp Gly Gln Gly Val
            100                 105                 110

ATG GTC ACA GTC TCC TCA                                              354
Met Val Thr Val Ser Ser
        115
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg
 1               5                  10                  15

Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
            20                  25                  30

Gly Met Ala Trp Val Arg Gln Ala Pro Thr Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Ser Ile Ser Thr Gly Gly Gly Asn Thr Tyr Tyr Arg Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Ser Glu Asp Thr Ala Thr Tyr Tyr Cys
                85                  90                  95

Ala Arg Pro Ser Tyr Ser Ser Tyr Phe Asp Tyr Trp Gly Gln Gly Val
            100                 105                 110

Met Val Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 402 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: LEW RAT
(F) TISSUE TYPE: Spleen, hyperimmunized
(G) CELL TYPE: Splenic lymphocyte

(vii) IMMEDIATE SOURCE:
(B) CLONE: HAR-1

(ix) FEATURE:
(A) NAME/KEY: CDS

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      (B) LOCATION: 1..402
      (D) OTHER INFORMATION: /product= "Immunoglobulin Variable
          Region"
          /standard_name= "Ig Kappa Chain Variable Region"
          /label= VK-Region
          /note= "Variable Region of HAR-1 Kappa Light
          Chain"

(ix) FEATURE:
      (A) NAME/KEY: misc_RNA
      (B) LOCATION: 1..60
      (D) OTHER INFORMATION: /standard_name= "Leader"
          /label= Leader

(ix) FEATURE:
      (A) NAME/KEY: misc_RNA
      (B) LOCATION: 61..129
      (D) OTHER INFORMATION: /standard_name= "Framework Region
          1"
          /label= FR-1
          /note= "Framework Region 1 of HAR-1 Kappa Light
          Chain Variable Region"

(ix) FEATURE:
      (A) NAME/KEY: misc_RNA
      (B) LOCATION: 130..180
      (D) OTHER INFORMATION: /standard_name= "CDR-1"
          /label= CDR-1
          /note= "Complimentarity Determining Region 1 of
          HAR-1 Kappa Light Chain Variable Region"

(ix) FEATURE:
      (A) NAME/KEY: misc_RNA
      (B) LOCATION: 181..225
      (D) OTHER INFORMATION: /standard_name= "Framework Region
          2"
          /label= FR-2
          /note= "Framework Region 2 of HAR-1 Kappa Light
          Chain Variable Region"

(ix) FEATURE:
      (A) NAME/KEY: misc_RNA
      (B) LOCATION: 226..246
      (D) OTHER INFORMATION: /standard_name= "CDR-2"
          /label= CDR-2
          /note= "Complimentarity Determining Region 2 of
          HAR-1 Kappa Light Chain Variable Region"

(ix) FEATURE:
      (A) NAME/KEY: misc_RNA
      (B) LOCATION: 247..342
      (D) OTHER INFORMATION: /standard_name= "Framework Region
          3"
          /label= FR-3
          /note= "Framework Region 3 of HAR-1 Kappa Light
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Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 343..366
(D) OTHER INFORMATION: /standard_name= "CDR-3"
/label= CDR-3
/note= "Complimentarity Determining Region 3 of HAR-1 Kappa Light Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 367..402
(D) OTHER INFORMATION: /standard_name= "Framework Region 4"
/label= FR-4
/note= "Framework Region 4 of HAR-1 Kappa Light Chain Variable Region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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ATG GAA TCA CAG ACA CAG GTC CTC ATG TCC CTG CTG CTC TGG GTA TCT        48
Met Glu Ser Gln Thr Gln Val Leu Met Ser Leu Leu Leu Trp Val Ser
 1               5                  10                  15

GGT ACC TGT GGG GAC ATT GTG ATG ACC CAG ACT CCA TCC TCC CAG GCT        96
Gly Thr Cys Gly Asp Ile Val Met Thr Gln Thr Pro Ser Ser Gln Ala
            20                  25                  30

GTG TCA GCA GGG GAG AAG GTC ACT ATG AGC TGC AAG TCC AGT CAG AGT       144
Val Ser Ala Gly Glu Lys Val Thr Met Ser Cys Lys Ser Ser Gln Ser
        35                  40                  45

CTT TTA TAC AAT GAA AAC AAA AAG AAC TAC TTG GCC TGG TAC CGG CAG       192
Leu Leu Tyr Asn Glu Asn Lys Lys Asn Tyr Leu Ala Trp Tyr Arg Gln
    50                  55                  60

AAA CCA GGG CAG TCT CCT AAA CTG CTG ATC TAC TGG GCA TCC ACT AGG       240
Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg
65                  70                  75                  80

GAA TCT GGG GTC CCT GAT CGC TTC ATA GGC AGT GGA TCT GGG ACA GAT       288
Glu Ser Gly Val Pro Asp Arg Phe Ile Gly Ser Gly Ser Gly Thr Asp
                85                  90                  95

TTC ACT CTG ACC ATC AGC AGT GTG CAG GCA GAA GAC CTG GCT GTT TAT       336
Phe Thr Leu Thr Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr
            100                 105                 110

TAC TGC CAG CAG TAC TAT AAC TTG TAC ACG TTT GGA GCT GGG ACC AAG       384
Tyr Cys Gln Gln Tyr Tyr Asn Leu Tyr Thr Phe Gly Ala Gly Thr Lys
        115                 120                 125

CTG GAA CTG AAA CGG GCT                                              402
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Leu Glu Leu Lys Arg Ala
130

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 134 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Glu Ser Gln Thr Gln Val Leu Met Ser Leu Leu Leu Trp Val Ser
1 5 10 15

Gly Thr Cys Gly Asp Ile Val Met Thr Gln Thr Pro Ser Ser Gln Ala
20 25 30

Val Ser Ala Gly Glu Lys Val Thr Met Ser Cys Lys Ser Ser Gln Ser
35 40 45

Leu Leu Tyr Asn Glu Asn Lys Lys Asn Tyr Leu Ala Trp Tyr Arg Gln
50 55 60

Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg
65 70 75 80

Glu Ser Gly Val Pro Asp Arg Phe Ile Gly Ser Gly Ser Gly Thr Asp
85 90 95

Phe Thr Leu Thr Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr
100 105 110

Tyr Cys Gln Gln Tyr Tyr Asn Leu Tyr Thr Phe Gly Ala Gly Thr Lys
115 120 125

Leu Glu Leu Lys Arg Ala
130

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature

(B) LOCATION: 1..20
(D) OTHER INFORMATION: /label= RVH1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TACAGCACTG CACAGACTCC 20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /label= RVH2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTGTCCTCAG ACCTCAGACT GTCC 24

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 440 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: LEW RAT
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(B) CLONE: VH1.1

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join(1..48, 159..440)
(D) OTHER INFORMATION: /label= VH-Segment
/note= "Rat Ig Germline VH1.1 Heavy Chain Variable Segment"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: join(1..48, 159..167)
(D) OTHER INFORMATION: /standard_name= "Leader"
/label= Leader

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 168..257
(D) OTHER INFORMATION: /standard_name= "Framework Region 1"
/label= FR-1
/note= "Framework Region 1 of Rat Ig Germline VH1.1 Heavy Chain Variable Segment"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 258..272
(D) OTHER INFORMATION: /standard_name= "CDR-1"
/label= CDR-1
/note= "Complimentarity Determining Region 1 of Rat Ig Germline VH1.1 Heavy Chain Variable Segment"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 273..315
(D) OTHER INFORMATION: /standard_name= "Framework Region 2"
/label= FR-2
/note= "Framework Region 2 of Rat Ig Germline VH1.1 Heavy Chain Variable Segment"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 316..365
(D) OTHER INFORMATION: /standard_name= "CDR-2"
/label= CDR-2
/note= "Complimentarity Determining Region 2 of Rat Ig Germline VH1.1 Heavy Chain Variable Segment"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 366..440
(D) OTHER INFORMATION: /standard_name= "Framework Region 3"
/label= FR-3
/note= "Framework Region 3 of Rat Ig Germline VH1.1 Heavy Chain Variable Segment"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATG GAC ATC AGG CTC AGC TTG GCT TTC CTT GTC CTT TTC ATA AAA GGT 48

```
Met Asp Ile Arg Leu Ser Leu Ala Phe Leu Val Leu Phe Ile Lys Gly
 1               5                  10                  15

AATTGATAAA AGTGTGATCA TCTCTGTTGT GTGCACATGA GAATAAGAAA GTTTATTTTG      108

TTTTGTTGTG TTAGTGATGG TTTTCTAACC AGTATTCTCT GTTTGCAGGT GTC CAG         164
                                                          Val Gln

TGT GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC TTA GTG CAG CCT GGA        212
Cys Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
     20                  25                  30

AGG TCC CTG AAA CTC TCC TGT GCA GCC TCA GGA TTC ACT TTC AGT AAC        260
Arg Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn
 35                  40                  45                  50

TAT GGC ATG GCC TGG GTC CGC CAG GCT CCA ACG AAG GGT CTG GAG TGG        308
Tyr Gly Met Ala Trp Val Arg Gln Ala Pro Thr Lys Gly Leu Glu Trp
                 55                  60                  65

GTC GCA TCC ATT AGT ACT GGT GGT GGT AAC ACT TAC TAT CGA GAC TCC        356
Val Ala Ser Ile Ser Thr Gly Gly Gly Asn Thr Tyr Tyr Arg Asp Ser
             70                  75                  80

GTG AAG GGC CGA TTC ACT ATC TCC AGA GAT AAT GCA AAA AAC ACC CTA        404
Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu
         85                  90                  95

TAC CTG CAA ATG GAC AGT CTG AGG TCT GAG GAC ACG                        440
Tyr Leu Gln Met Asp Ser Leu Arg Ser Glu Asp Thr
    100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Asp Ile Arg Leu Ser Leu Ala Phe Leu Val Leu Phe Ile Lys Gly
 1               5                  10                  15

Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln
            20                  25                  30

Pro Gly Arg Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45

Ser Asn Tyr Gly Met Ala Trp Val Arg Gln Ala Pro Thr Lys Gly Leu
```

```
     50                  55                  60

Glu Trp Val Ala Ser Ile Ser Thr Gly Gly Gly Asn Thr Tyr Tyr Arg
 65                  70                  75                  80

Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
                 85                  90                  95

Thr Leu Tyr Leu Gln Met Asp Ser Leu Arg Ser Glu Asp Thr
           100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 348 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: LEW RAT
(F) TISSUE TYPE: Spleen, hyperimmunized
(G) CELL TYPE: Splenic lymphocyte

(vii) IMMEDIATE SOURCE:
(B) CLONE: 9D6

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..348
(D) OTHER INFORMATION: /product= "Immunoglobulin Variable Region"
/standard_name= "Ig Heavy Chain Variable Region"
/label= VH-Region
/note= "Variable Region of 9D6 Heavy Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..294
(D) OTHER INFORMATION: /standard_name= "Ig Heavy Chain Variable Segment"
/label= VH-Segment
/note= "Variable Segment of 9D6 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 295..303
(D) OTHER INFORMATION: /standard_name= "Ig Heavy Chain Diversity Segment"
/label= D-Segment

/note= "Diversity Segment of 9D6 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 304..348
(D) OTHER INFORMATION: /standard_name= "Ig Heavy Chain Joining Segment"
/label= JH-Segment
/note= "Joining Segment of 9D6 Heavy Chain Variable Region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
GAG GTG AAA CTT GTC GAG TCT GGA GGT GGC CTG GTG CAA CCT GGA AGA        48
Glu Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg
  1               5                  10                  15

TCC TTG AAA CTC TCC TGT GCA GCC TCT GGA TTC AAT TTT AAT GAT TAC        96
Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Asn Phe Asn Asp Tyr
             20                  25                  30

TGG ATG GGC TGG GTC CGG CAG GCT CCA GGG AAG GGG CTA GAA TGG ATT       144
Trp Met Gly Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
         35                  40                  45

GGA GAA ATT AAT AAG GAT AGC AGT ACA ATA AAC TAT ACT CCA TCC TTG       192
Gly Glu Ile Asn Lys Asp Ser Ser Thr Ile Asn Tyr Thr Pro Ser Leu
     50                  55                  60

AAG GAT AAA TTC ACC ATC TCC AGA GAC AAT GCC CAA AAC ACT CTG TAC       240
Lys Asp Lys Phe Thr Ile Ser Arg Asp Asn Ala Gln Asn Thr Leu Tyr
 65                  70                  75                  80

CTG CAA ATG AGC AAA CTG GGA TCT GAG GAC ACG GCC ATT TAT TAC TGT       288
Leu Gln Met Ser Lys Leu Gly Ser Glu Asp Thr Ala Ile Tyr Tyr Cys
                 85                  90                  95

GCA AAA GCA ACT GGG AGC TTT GAT TAC TGG GGC CAA GGA GTC ATG GTC       336
Ala Lys Ala Thr Gly Ser Phe Asp Tyr Trp Gly Gln Gly Val Met Val
            100                 105                 110

ACA GTC TCC TCA                                                      348
Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Glu Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg
 1               5                  10                  15

Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Asn Phe Asn Asp Tyr
            20                  25                  30

Trp Met Gly Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Glu Ile Asn Lys Asp Ser Ser Thr Ile Asn Tyr Thr Pro Ser Leu
    50                  55                  60

Lys Asp Lys Phe Thr Ile Ser Arg Asp Asn Ala Gln Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Ser Lys Leu Gly Ser Glu Asp Thr Ala Ile Tyr Tyr Cys
                85                  90                  95

Ala Lys Ala Thr Gly Ser Phe Asp Tyr Trp Gly Gln Gly Val Met Val
            100                 105                 110

Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /label= RCM1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACTGGCTCTG TGGTGAAGCC 20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..32
(D) OTHER INFORMATION: /label= SAX

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGAATTCGGG CCCTCGAGGC CTCTAGAATT CG 32

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..23
(D) OTHER INFORMATION: /label= RCM3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTTCTGGTAG TTCCAGGAGA AGG 23

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /label= RCK1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTGAAGCTCT TGACGACGGG 20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /label= RCK3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCTAACTGTT CCGTGGATGG TGGG 24

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 345 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: LEW RAT
(F) TISSUE TYPE: Spleen, hyperimmunized
(G) CELL TYPE: Splenic lymphocyte

(vii) IMMEDIATE SOURCE:
(B) CLONE: HA75D8F1

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..345
(D) OTHER INFORMATION: /product= "Immunoglobulin Variable Region"
/standard_name= "Ig Heavy Chain Variable Region"
/label= VH-Region
/note= "Variable Region of HA75D8F1 Heavy Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..291

(D) OTHER INFORMATION: /standard_name= "Ig Heavy Chain Variable Segment"
/label= VH-Segment
/note= "Variable Segment of HA75D8F1 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 292..312
(D) OTHER INFORMATION: /standard_name= "Ig Heavy Chain Diversity Segment"
/label= D-Segment
/note= "Diversity Segment of HA75D8F1 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 313..345
(D) OTHER INFORMATION: /standard_name= "Ig Heavy Chain Joining Segment"
/label= JH-Segment
/note= "Joining Segment of HA75D8F1 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..90
(D) OTHER INFORMATION: /standard_name= "Framework Region 1"
/label= FR-1
/note= "Framework Region 1 of HA75D8F1 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 91..105
(D) OTHER INFORMATION: /standard_name= "CDR-1"
/label= CDR-1
/note= "Complimentarity Determining Region 1 of HA75D8F1 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 106..151
(D) OTHER INFORMATION: /standard_name= "Framework Region 2"
/label= FR-2
/note= "Framework Region 2 of HA75D8F1 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 152..198
(D) OTHER INFORMATION: /standard_name= "CDR-2"
/label= CDR-2

/note= "Complimentarity Determining Region 2 of HA75D8F1 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 199..291
(D) OTHER INFORMATION: /standard_name= "Framework Region 3"
/label= FR-3
/note= "Framework Region 3 of HA75D8F1 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 292..312
(D) OTHER INFORMATION: /standard_name= "CDR-3"
/label= CDR-3
/note= "Complimentarity Determining Region 3 of HA75D8F1 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 313..345
(D) OTHER INFORMATION: /standard_name= "Framework Region 4"
/label= FR-4
/note= "Framework Region 4 of HA75D8F1 Heavy Chain Variable Region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
GAG GTG AAG CTG CAG GAG TCA GGA CCT GGT CTG GTA CAG CCC TCA CAG        48
Glu Val Lys Leu Gln Glu Ser Gly Pro Gly Leu Val Gln Pro Ser Gln
 1               5                  10                  15

ACC CTG TCC CTC ACC TGC ACT GTC TCT GGG TTC TCA CTA AAC AAC TAT        96
Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Ser Leu Asn Asn Tyr
            20                  25                  30

GGT GTG ATC TGG GTT CGC CAG CCT CCA GGA AAG GGT CTG GAG TGG ATG       144
Gly Val Ile Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Met
        35                  40                  45

GGA ATA ATT TGG AAT AAT GGA AAT ACA AAT TAT AAT TCA GCT CTC AAA       192
Gly Ile Ile Trp Asn Asn Gly Asn Thr Asn Tyr Asn Ser Ala Leu Lys
    50                  55                  60

TCC CGA CTG AGC ATC AGC AGG GAC ACC TCC AAG AGC CAA GTT TTC TTA       240
Ser Arg Leu Ser Ile Ser Arg Asp Thr Ser Lys Ser Gln Val Phe Leu
65                  70                  75                  80

AAA ATG AAC AAT CTG CAA ACT GAA GAC ACG GCC ATG TAC TTC TGT GCC       288
Lys Met Asn Asn Leu Gln Thr Glu Asp Thr Ala Met Tyr Phe Cys Ala
                85                  90                  95
```

```
AGA GGA GGA GTG GGG TTT GAT TTC TGG GGC CAA GGA GTC ATG GTC ACA      336
Arg Gly Gly Val Gly Phe Asp Phe Trp Gly Gln Gly Val Met Val Thr
            100                 105                 110

GTC TCC TCA                                                      345
Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 115 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
Glu Val Lys Leu Gln Glu Ser Gly Pro Gly Leu Val Gln Pro Ser Gln
  1               5                  10                  15

Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Ser Leu Asn Asn Tyr
             20                  25                  30

Gly Val Ile Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Met
         35                  40                  45

Gly Ile Ile Trp Asn Asn Gly Asn Thr Asn Tyr Asn Ser Ala Leu Lys
     50                  55                  60

Ser Arg Leu Ser Ile Ser Arg Asp Thr Ser Lys Ser Gln Val Phe Leu
 65                  70                  75                  80

Lys Met Asn Asn Leu Gln Thr Glu Asp Thr Ala Met Tyr Phe Cys Ala
                 85                  90                  95

Arg Gly Gly Val Gly Phe Asp Phe Trp Gly Gln Gly Val Met Val Thr
            100                 105                 110

Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 357 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: LEW RAT
(F) TISSUE TYPE: Spleen, hyperimmunized
(G) CELL TYPE: Splenic lymphocyte

(vii) IMMEDIATE SOURCE:
(B) CLONE: IH21H7

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..357
(D) OTHER INFORMATION: /product= "Immunoglobulin Variable Region"
/standard_name= "Ig Heavy Chain Variable Region"
/label= VH-Region
/note= "Variable Region of IH21H7 Heavy Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..291
(D) OTHER INFORMATION: /standard_name= "Ig Heavy Chain Variable Segment"
/label= VH-Segment
/note= "Variable Segment of IH21H7 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 292..312
(D) OTHER INFORMATION: /standard_name= "Ig Heavy Chain Diversity Segment"
/label= D-Segment
/note= "Diversity Segment of IH21H7 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 313..357
(D) OTHER INFORMATION: /standard_name= "Ig Heavy Chain Joining Segment"
/label= JH-Segment
/note= "Joining Segment of IH21H7 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..90
(D) OTHER INFORMATION: /standard_name= "Framework Region 1"
/label= FR-1
/note= "Framework Region 1 of IH21H7 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA

(B) LOCATION: 91..105
(D) OTHER INFORMATION: /standard_name= "CDR-1"
/label= CDR-1
/note= "Complimentarity Determining Region 1 of IH21H7 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 106..151
(D) OTHER INFORMATION: /standard_name= "Framework Region 2"
/label= FR-2
/note= "Framework Region 2 of IH21H7 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 152..198
(D) OTHER INFORMATION: /standard_name= "CDR-2"
/label= CDR-2
/note= "Complimentary Determining Region 2 of IH21H7 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 199..291
(D) OTHER INFORMATION: /standard_name= "Framework Region 3"
/label= FR-3
/note= "Framework Region 3 of IH21H7 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 292..324
(D) OTHER INFORMATION: /standard_name= "CDR-3"
/label= CDR-3
/note= "Complimentarity Determining Region 3 of IH21H7 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 325..357
(D) OTHER INFORMATION: /standard_name= "Framework Region 4"
/label= FR-4
/note= "Framework Region 4 of IH21H7 Heavy Chain Variable Region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
GAG GTC AAG CTG CAG CAG TCA GGA CCT GGC CTG GTG CAG CCC TCA CAG        48
Glu Val Lys Leu Gln Gln Ser Gly Pro Gly Leu Val Gln Pro Ser Gln
 1               5                  10                  15
```

```
ACC CTG TCT CTC ACC TGC ACT GTC TCT GGG TTC TCA TTA ACC AAC TAT          96
Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Ser Leu Thr Asn Tyr
             20                  25                  30

CAT GTG CAC TGG GTT CGA CAG CCT CCA GGA AAA GGT CTG GAG TGG ATG         144
His Val His Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Met
         35                  40                  45

GGA GTC ATG TGG GGT GAT GGA GAC ACA TCA TGT AAT TCA GCT CTC AAA         192
Gly Val Met Trp Gly Asp Gly Asp Thr Ser Cys Asn Ser Ala Leu Lys
     50                  55                  60

TCC CGA CTG AGC ATC AGC AGG GAC ACC TCC AAG AGC CAA GTT TTC TTA         240
Ser Arg Leu Ser Ile Ser Arg Asp Thr Ser Lys Ser Gln Val Phe Leu
 65                  70                  75                  80

AAA TTG AGC AGT CTG CAA ACT GAA GAC ACA GCC ACT TAC TAC TGT GCC         288
Lys Leu Ser Ser Leu Gln Thr Glu Asp Thr Ala Thr Tyr Tyr Cys Ala
                 85                  90                  95

AGA CTC CCT AGG GGG AAG GGA CCC CAC TTT GAT TAC TGG GGC CAA GGA         336
Arg Leu Pro Arg Gly Lys Gly Pro His Phe Asp Tyr Trp Gly Gln Gly
            100                 105                 110

GTC ATG GTC ACA GTC TCC TCA                                             357
Val Met Val Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Glu Val Lys Leu Gln Gln Ser Gly Pro Gly Leu Val Gln Pro Ser Gln
  1               5                  10                  15

Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Ser Leu Thr Asn Tyr
             20                  25                  30

His Val His Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Met
         35                  40                  45

Gly Val Met Trp Gly Asp Gly Asp Thr Ser Cys Asn Ser Ala Leu Lys
     50                  55                  60

Ser Arg Leu Ser Ile Ser Arg Asp Thr Ser Lys Ser Gln Val Phe Leu
 65                  70                  75                  80
```

```
Lys Leu Ser Ser Leu Gln Thr Glu Asp Thr Ala Thr Tyr Tyr Cys Ala
                85                  90                  95

Arg Leu Pro Arg Gly Lys Gly Pro His Phe Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Val Met Val Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 354 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: LEW RAT
(F) TISSUE TYPE: Spleen, hyperimmunized
(G) CELL TYPE: Splenic lymphocyte

(vii) IMMEDIATE SOURCE:
(B) CLONE: ID12CF2

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..354
(D) OTHER INFORMATION: /product= "Immunoglobulin Variable Region"
/standard_name= "Ig Heavy Chain Variable Region"
/label= VH-Region
/note= "Variable Region of ID12CF2 Heavy Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..90
(D) OTHER INFORMATION: /standard_name= "Framework Region 1"
/label= FR-1
/note= "Framework Region 1 of ID12CF2 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 91..105
(D) OTHER INFORMATION: /standard_name= "CDR-1"
/label= CDR-1
/note= "Complimentarity Determining Region 1 of ID12CF2 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 106..147
(D) OTHER INFORMATION: /standard_name= "Framework Region 2"
/label= FR-2
/note= "Framework Region 2 of ID12CF2 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 148..198
(D) OTHER INFORMATION: /standard_name= "CDR-2"
/label= CDR-2
/note= "Complimentarity Determining Region 2 of ID12CF2 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 199..294
(D) OTHER INFORMATION: /standard_name= "Framework Region 3"
/label= FR-3
/note= "Framework Region 3 of ID12CF2 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 295..321
(D) OTHER INFORMATION: /standard_name= "CDR-3"
/label= CDR-3
/note= "Complimentarity Determining Region 3 of ID12CF2 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 322..354
(D) OTHER INFORMATION: /standard_name= "Framework Region 4"
/label= FR-4
/note= "Framework Region 4 of ID12CF2 Heavy Chain Variable Region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC TTA GTG CAG CCT GGA AGA        48
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg
 1               5                  10                  15

TCC CTG AAA CTC TCC TGT GCA GCC TCA GGA TTC ACT TTC AGT AAC TAT        96
Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
            20                  25                  30

GGC ATG GCT TGG GTC CGC CAG GCT CCA ACG AAG GGT CTG GAG TGG GTC       144
```

```
Gly Met Ala Trp Val Arg Gln Ala Pro Thr Lys Gly Leu Glu Trp Val
         35                  40                  45

GCA TCC ATT AGT ACT GGT GGT GGT AAC ACT TAC TAT CGA GAC TCC GTG        192
Ala Ser Ile Ser Thr Gly Gly Gly Asn Thr Tyr Tyr Arg Asp Ser Val
     50                  55                  60

AAG GGC CGA TTC ACT ATC TCC AGA GAT AAT GCA AAA AAC ACC CTA TAC        240
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
 65                  70                  75                  80

CTG CAA ATG GAC AGT CTG AGG TCT GAG GAC ACG GCC ACT TAT TAC TGT        288
Leu Gln Met Asp Ser Leu Arg Ser Glu Asp Thr Ala Thr Tyr Tyr Cys
                 85                  90                  95

GCA AGA CCT TCC TAT AGC AGC TAC TTT GAT TAC TGG GGC CAA GGA GTC        336
Ala Arg Pro Ser Tyr Ser Ser Tyr Phe Asp Tyr Trp Gly Gln Gly Val
            100                 105                 110

ATG GTC ACA GTC TCC TCA                                               354
Met Val Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg
  1               5                  10                  15

Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
            20                  25                  30

Gly Met Ala Trp Val Arg Gln Ala Pro Thr Lys Gly Leu Glu Trp Val
         35                  40                  45

Ala Ser Ile Ser Thr Gly Gly Gly Asn Thr Tyr Tyr Arg Asp Ser Val
     50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
 65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Ser Glu Asp Thr Ala Thr Tyr Tyr Cys
                 85                  90                  95

Ala Arg Pro Ser Tyr Ser Ser Tyr Phe Asp Tyr Trp Gly Gln Gly Val
            100                 105                 110
```

Met Val Thr Val Ser Ser
115

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 348 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: LEW RAT
(F) TISSUE TYPE: Spleen, hyperimmunized
(G) CELL TYPE: Splenic lymphocyte

(vii) IMMEDIATE SOURCE:
(B) CLONE: FC2EG11

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..348
(D) OTHER INFORMATION: /product= "Immunoglobulin Variable Region"
/standard_name= "Ig Heavy Chain Variable Region"
/label= VH-Region
/note= "Variable Region of FC2EG11 Heavy Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..90
(D) OTHER INFORMATION: /standard_name= "Framework Region 1"
/label= FR-1
/note= "Framework Region 1 of FC2EG11 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 91..105
(D) OTHER INFORMATION: /standard_name= "CDR-1"
/label= CDR-1
/note= "Complimentarity Determining Region 1 of FC2EG11 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 106..147
(D) OTHER INFORMATION: /standard_name= "Framework Region 2"
/label= FR-2

/note= "Framework Region 2 of FC2EG11 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 148..198
(D) OTHER INFORMATION: /standard_name= "CDR-2"
/label= CDR-2
/note= "Complimentarity Determining Region 2 of FC2EG11 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 199..294
(D) OTHER INFORMATION: /standard_name= "Framework Region 3"
/label= FR-3
/note= "Framework Region 3 of FC2EG11 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 295..315
(D) OTHER INFORMATION: /standard_name= "CDR-3"
/label= CDR-3
/note= "Complimentarity Determining Region 3 of FC2EG11 Heavy Chain Variable Region"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 316..348
(D) OTHER INFORMATION: /standard_name= "Framework Region 4"
/label= FR-4
/note= "Framework Region 4 of FC2EG11 Heavy Chain Variable Region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC TTA GTG CAG CCT GGA AGA        48
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg
 1               5                  10                  15

TCC ATG AAA CTC TCC TGT GCA GCC TCA GGA TTC ACT TTC AGT AAC TAT        96
Ser Met Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
            20                  25                  30

TAC ATG GCC TGG GTC CGC CAG GCT CCA ACG AAG GGT CTG GAG TGG GTC       144
Tyr Met Ala Trp Val Arg Gln Ala Pro Thr Lys Gly Leu Glu Trp Val
        35                  40                  45

GCA TCC ATT AGT ACT GGT GGT GGT AAC ACT TAC TAT CGA GAC TCC GTG       192
Ala Ser Ile Ser Thr Gly Gly Gly Asn Thr Tyr Tyr Arg Asp Ser Val
    50                  55                  60
```

```
AAG GGC CGA TTC ACT ATC TCC AGA GAT AAT GCA AAA AAC ACC CTA TAC        240
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
 65                  70                  75                  80

CTG CAA ATG GAC AGT CTG AGG TCT GAG GAC ACG GCC ACT TAT TAC TGT        288
Leu Gln Met Asp Ser Leu Arg Ser Glu Asp Thr Ala Thr Tyr Tyr Cys
                85                  90                  95

GCA AGA GGG GAG GCC TAC TTT GAT TAC TGG GGC CAA GGA GTC ATG GTC        336
Ala Arg Gly Glu Ala Tyr Phe Asp Tyr Trp Gly Gln Gly Val Met Val
            100                 105                 110

ACA GTC TCC TCA                                                       348
Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg
  1               5                  10                  15

Ser Met Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
            20                  25                  30

Tyr Met Ala Trp Val Arg Gln Ala Pro Thr Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Ser Ile Ser Thr Gly Gly Gly Asn Thr Tyr Tyr Arg Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
 65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Ser Glu Asp Thr Ala Thr Tyr Tyr Cys
                85                  90                  95

Ala Arg Gly Glu Ala Tyr Phe Asp Tyr Trp Gly Gln Gly Val Met Val
            100                 105                 110

Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..18
(D) OTHER INFORMATION: /label= VH1
/note= "VH1 Family Specific Primer for Human Heavy Chain Ig"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCATGGACTG GACCTGGA 18

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /label= VH2
/note= "VH2 Family Specific Primer for Human Heavy Chain Ig"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ATGGACATAC TTTGTTCCAC 20

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /label= VH3
/note= "VH3 Family Specific Primer for Human Heavy Chain Ig"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CCATGGAGTT TGGGCTGAGC 20

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /label= VH4
/note= "VH4 Family Specific Primer for Human Heavy Chain Ig"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ATGAAACACC TGTGGTTCTT 20

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /label= VH5
/note= "VH5 Family Specific Primer for Human Heavy Chain Ig"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ATGGGGTCAA CCGCCATCCT 20

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /label= VH6
/note= "VH6 Family Specific Primer for Human Heavy Chain Ig"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

ATGTCTGTCT CCTTCCTCAT 20

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /label= HCM2
/note= "HCM2 Isotype Specific Primer for Human Heavy Chain Ig"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TCACAGGAGA CGAGGGGGAA 20

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..100
(D) OTHER INFORMATION: /label= Not1-Linker
/note= "Oligonucleotide Linker Encoding Not 1 Restriction Site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

AATTAACCCT CACTAAAGGG AACAAAAGCT GGAGCTTGAA TTCTTAACTA CTCGCCAAGG 60

AGACAGTCAT AATGAAATAC CTATTGCCTA CGGCGGCCGC 100

WE CLAIM:

1. A method of inhibiting rejection of a xenograft from a donor animal by a recipient animal, comprising:

modifying antigen expressed by cells of the xenograft, without causing lysis of the cells, to inhibit binding of recipient anti-donor xenograft antibody to said antigen, wherein said antigen present in unmodified form induces an antibody-mediated immune response in the recipient animal.

2. The method of claim 1, wherein said modifying comprises contacting non-lytic, anti-donor xenograft antibody material with said antigen for a time, at a temperature, and at a pH suitable to bind the antibody material to the antigen, wherein said anti-donor xenograft antibody material is characterized as immunoreactive with antigen expressed by endothelial cells of the xenograft and capable of inhibiting antibody-mediated rejection of the xenograft by a recipient animal.

3. A method for transplanting a xenograft in a patient, said method comprising:

contacting said xenograft, prior to transplantation, with anti-donor xenograft antibody material for a time, at a temperature, and at a pH suitable to allow said antibody material to immunoreact with antigen expressed by said xenograft, and then

transplanting said xenograft.

4. Isolated and substantially purified anti-donor xenograft antibody that is immunoreactive with antigen expressed by endothelial cells of a donor xenograft and is capable of inducing antibody-mediated rejection of the xenograft by a recipient animal.

5. Isolated and substantially purified anti-donor xenograft antibody material characterized as being immunoreactive with antigen expressed by endothelial cells of a donor xenograft and capable of inhibiting antibody-mediated rejection of the donor xenograft by a recipient animal.

6. The antibody material of claim 5, further comprising:

(a) at least one polypeptide encoded by a nucleic acid sequence including the sequence defined by nucleic acid residues 1 through 345 of SEQ ID NO: 18;

(b) at least one human immunoglobulin light chain; and wherein said donor xenograft is tissue from a pig.

7. The antibody material of claim 5, further comprising:

(a) at least one polypeptide encoded by a nucleic acid sequence including the sequence defined by nucleic acid residues 1 through 357 of SEQ ID NO: 20;

(b) at least one human immunoglobulin light chain; and wherein said donor xenograft is tissue from a pig.

8. Isolated and purified polynucleotide comprising a nucleic acid sequence encoding the polypeptide defined by amino acid residues 1 through 420 of SEQ ID NO: 1.

9. Isolated and purified polypeptide encoded by the polynucleotide of claim 8.

10. Isolated and purified polynucleotide comprising a nucleic acid sequence encoding the polypeptide defined by amino acid residues 1 through 402 of SEQ ID NO: 5.

11. Isolated and purifies polypeptide encoded by the polynucleotide of claim 10.

12. Isolated and purified polynucleotide comprising a nucleic acid sequence encoding the polypeptide defined by amino acid residues 1 through 354 of SEQ ID NO: 3.

13. Isolated and purified polypeptide encoded by the polynucleotide of claim 12.

14. Isolated and purified polynucleotide comprising a nucleic acid sequence encoding the polypeptide defined by amino acid residues 1 through 345 of SEQ ID NO: 18.

15. Isolated and purified polypeptide encoded by the polynucleotide of claim 14.

16. Isolated and purified polynucleotide comprising a nucleic acid sequence encoding the polypeptide defined by amino acid residues 1 through 357 of SEQ ID NO: 20.

17. Isolated and purified polypeptide encoded by the polynucleotide of claim 16.

18. Recombinant anti-donor xenograft antibody material characterized as immunoreactive with antigen expressed by endothelial cells of a donor xenograft and capable of inhibiting antibody-mediated rejection of the donor xenograft by a recipient animal, wherein said antibody material comprises at least one immunoglobulin light chain polypeptide and at least one immunoglobulin heavy chain variable region polypeptide.

19. The antibody material of claim 18, wherein said immunoglobulin light chain polypeptide is human.

20. The antibody material of claim 19, wherein said immunoglobulin heavy chain variable region polypeptide is human.

21. The antibody material of claim 18, wherein said immunoglobulin heavy chain variable region polypeptide is human.

22. Nucleic acid encoding the antibody material of claim 21.

23. A vector comprising the nucleic acid of claim 22.

24. The vector of claim 23, wherein said vector is a phagemid.

25. A cell containing the vector of claim 24.

26. A method of isolating antigen expressed by endothelial cells of a xenograft and which is characterized as inducing antibody-mediated rejection of the xenograft by a recipient animal, said method comprising:

contacting anti-donor xenograft antibody with endothelial cell membrane lysate of the xenograft for a time and at a temperature and pH suitable to form an immunecomplex comprising said antibody;

separating said immune-complex from said non-complexed endothelial cell membrane lysate;

separating said anti-donor xenograft antibody material from said antigen.

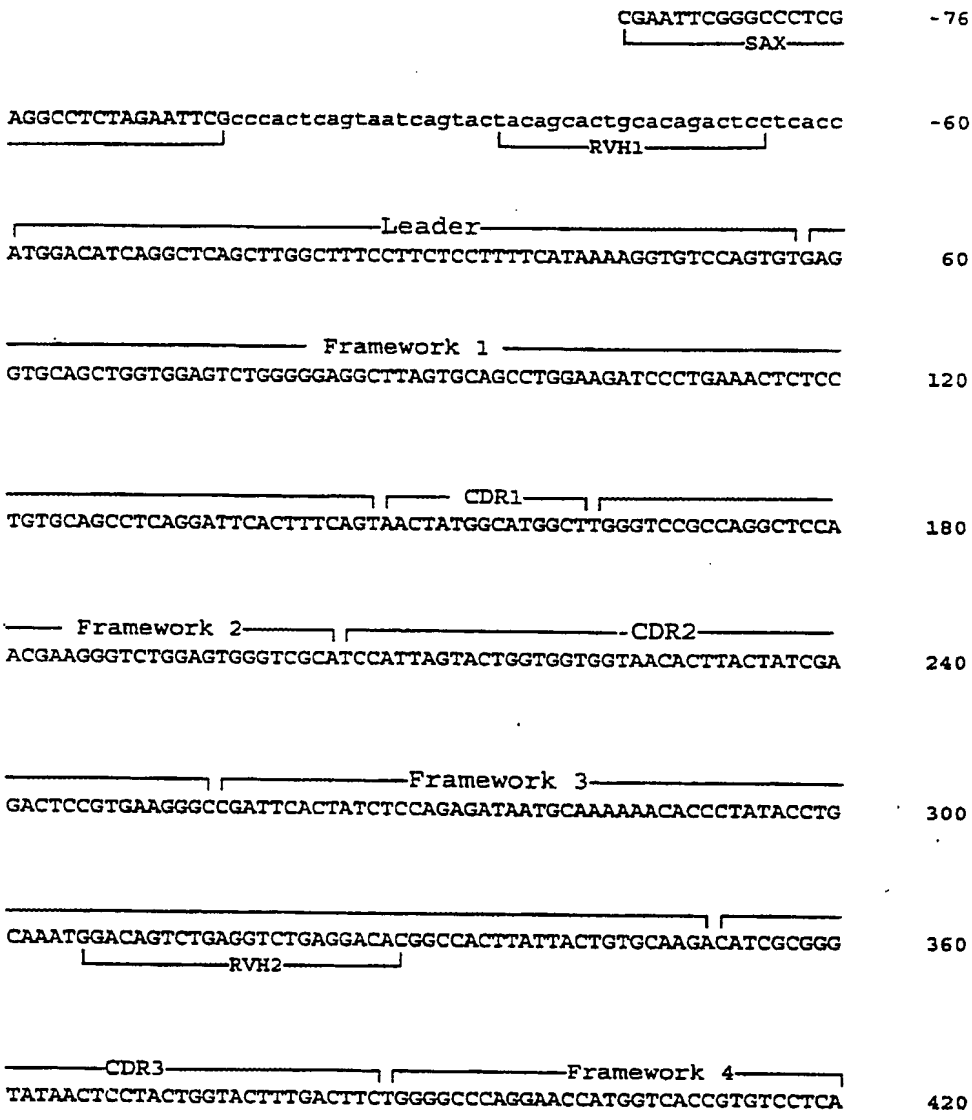
CGAATTCGGGCCCTCG -76
SAX
AGGCCTCTAGAATTCGcccactcagtaatcagtactacagcactgcacagactcctcacc -60
RVH1
Leader
ATGGACATCAGGCTCAGCTTGGCTTTCCTTCTCCTTTTCATAAAAGGTGTCCAGTGTGAG 60
Framework 1
GTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGCAGCCTGGAAGATCCCTGAAACTCTCC 120
CDR1
TGTGCAGCCTCAGGATTCACTTTCAGTAACTATGGCATGGCTTGGGTCCGCCAGGCTCCA 180
Framework 2
CDR2
ACGAAGGGTCTGGAGTGGGTCGCATCCATTAGTACTGGTGGTGGTAACACTTACTATCGA 240
Framework 3
GACTCCGTGAAGGGCCGATTCACTATCTCCAGAGATAATGCAAAAAACACCCTATACCTG 300
CAAATGGACAGTCTGAGGTCTGAGGACACGGCCACTTATTACTGTGCAAGACATCGCGGG 360
RVH2
CDR3
Framework 4
TATAACTCCTACTGGTACTTTGACTTCTGGGGCCCAGGAACCATGGTCACCGTGTCCTCA 420

**FIGURE 1**

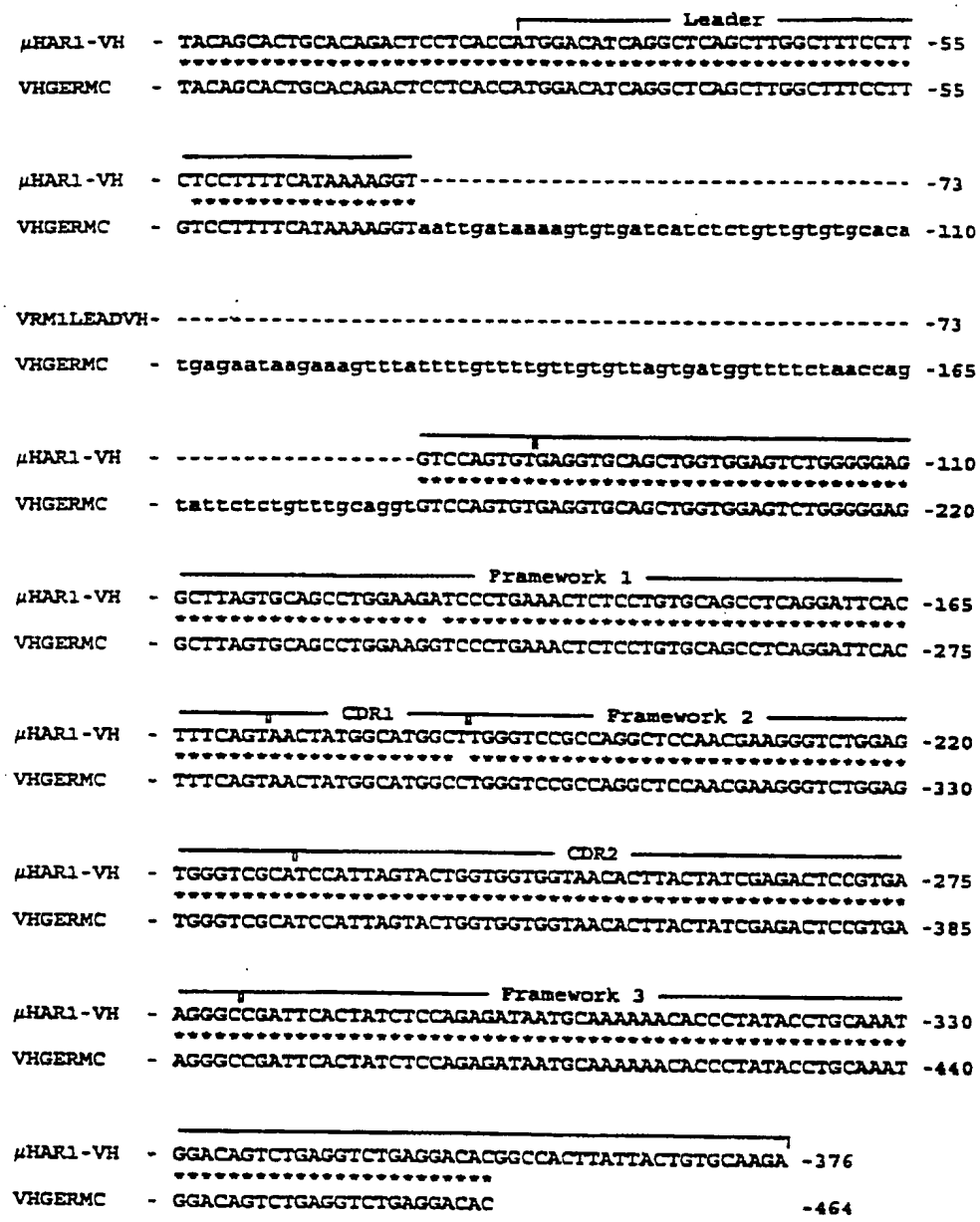
Leader
μHAR1-VH - TACAGCACTGCACAGACTCCTCACCATGGACATCAGGCTCAGCTTGGCTTTCCTT -55
VHGERMC - TACAGCACTGCACAGACTCCTCACCATGGACATCAGGCTCAGCTTGGCTTTCCTT -55
μHAR1-VH - CTCCTTTTCATAAAAGGT- -73
VHGERMC - GTCCTTTTCATAAAAGGTaattgataaaagtgtgatcatctctgttgtgtgcaca -110
VRM1LEADVH- ---------- -73
VHGERMC - tgagaataagaaagtttatttgttttgttgtgttagtgatggttttctaaccag -165
μHAR1-VH - ----------GTCCAGTGTGAGGTGCAGCTGGTGGAGTCTGGGGGAG -110
VHGERMC - tattctctgtttgcaggtGTCCAGTGTGAGGTGCAGCTGGTGGAGTCTGGGGGAG -220
Framework 1
μHAR1-VH - GCTTAGTGCAGCCTGGAAGATCCCTGAAACTCTCCTGTGCAGCCTCAGGATTCAC -165
VHGERMC - GCTTAGTGCAGCCTGGAAGGTCCCTGAAACTCTCCTGTGCAGCCTCAGGATTCAC -275
CDR1
Framework 2
μHAR1-VH - TTTCAGTAACTATGGCATGGCTTGGGTCCGCCAGGCTCCAACGAAGGGTCTGGAG -220
VHGERMC - TTTCAGTAACTATGGCATGGCCTGGGTCCGCCAGGCTCCAACGAAGGGTCTGGAG -330
CDR2
μHAR1-VH - TGGGTCGCATCCATTAGTACTGGTGGTGGTAACACTTACTATCGAGACTCCGTGA -275
VHGERMC - TGGGTCGCATCCATTAGTACTGGTGGTGGTAACACTTACTATCGAGACTCCGTGA -385
Framework 3
μHAR1-VH - AGGGCCGATTCACTATCTCCAGAGATAATGCAAAAAACACCCTATACCTGCAAAT -330
VHGERMC - AGGGCCGATTCACTATCTCCAGAGATAATGCAAAAAACACCCTATACCTGCAAAT -440
μHAR1-VH - GGACAGTCTGAGGTCTGAGGACACGGCCACTTATTACTGTGCAAGA -376
VHGERMC - GGACAGTCTGAGGTCTGAGGACAC -464

FIGURE 2

```
HA75D8F1-   AGGTGAAGCTGCAGGAGTCAGGACCTGGTCTGGTACAGCCCTCACAGACCCTGTC   55
            **** ********* ************ ***** ********************
IH21H7-     AGGTCAAGCTGCAGCAGTCAGGACCTGGCCTGGTGCAGCCCTCACAGACCCTGTC   55

HA75D8F1-   CCTCACCTGCACTGTCTCTGGGTTCTCACTAAACAACTATGGTGTGATCTGGGTT   110
            **************************** *** *******  ****  *******
IH21H7-     TCTCACCTGCACTGTCTCTGGGTTCTCATTAACCAACTATCATGTGCACTGGGTT   110

HA75D8F1-   CGCCAGCCTCCAGGAAAGGGTCTGGAGTGGATGGGAATAATTTGGAATAATGGAA   165
            ** ************** ***************** * ** ***  * *****
IH21H7-     CGACAGCCTCCAGGAAAAGGTCTGGAGTGGATGGGAGTCATGTGGGGTGATGGAG   165

HA75D8F1-   ATACAAATTATAATTCAGCTCTCAAATCCCGACTGAGCATCAGCAGGGACACCTC   220
            * ***   * ********************************************
IH21H7-     ACACATCATGTAATTCAGCTCTCAAATCCCGACTGAGCATCAGCAGGGACACCTC   220

HA75D8F1-   CAAGAGCCAAGTTTTCTTAAAAATGAACAATCTGCAAACTGAAGACACGGCCATG   275
            ********************* *** ** ****************** ****
IH21H7-     CAAGAGCCAAGTTTTCTTAAAATTGAGCAGTCTGCAAACTGAAGACACAGCCACT   275

HA75D8F1-   TACTTCTGTGCCAGA-------GGAGGAGTGGGG-----TTTGATTTCTGGGGCC   318
            **** **********       ** ***  **       ******* ********
IH21H7-     TACTACTGTGCCAGACTCCCTAGGGGGAAGGGACCCCACTTTGATTACTGGGGCC   330

HA75D8F1-   AAGGAGTCATGGTCACAGTCTCCTCAGA                              346
            ***************************
IH21H7-     AAGGAGTCATGGTCACAGTCTCCTCAGA                              358
```

FIGURE 3

# Nucleotide Sequence of the $V_H$ Chain of HA75D8F1 Compared to its Germline Gene

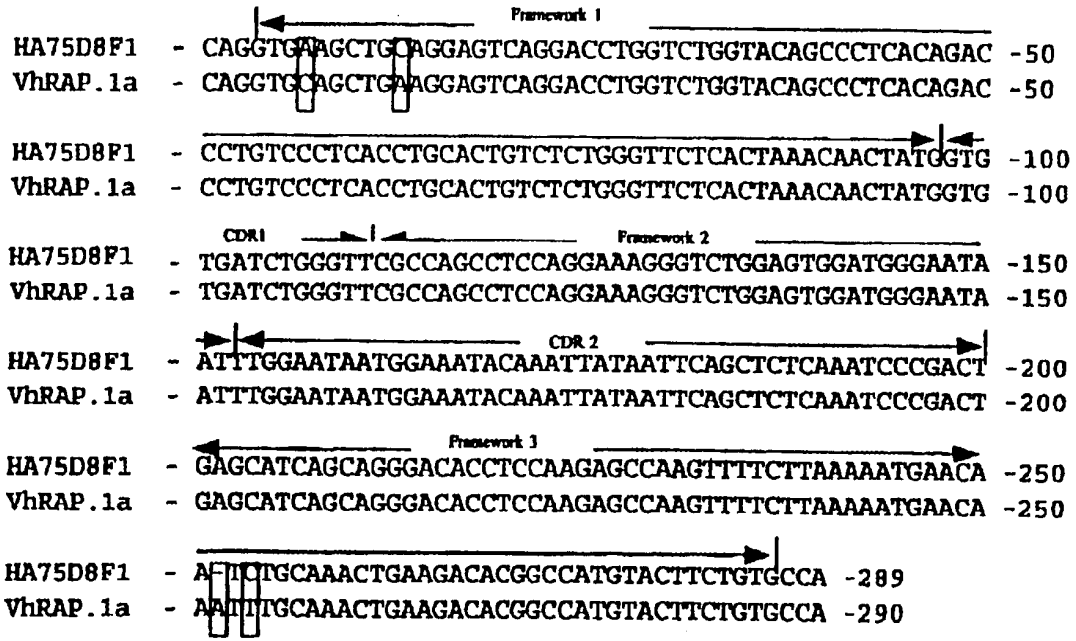


FIGURE 4

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/06804

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, DERWENT WPI, CHEM AB, APS; search terms: author names, xenograft, endothelial cells, porcine, pig, har-1, antibody, inhibit, cell surface, inhibit, transplantation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US, 5,283,058 A (D. FAUSTMAN) 01 February 1994, see entire document. | 1-26 |
| Y | WU et al. Distribution of xenogeneic target antigens detected by a monoclonal antibody capable of inducing hyperacute rejection of hamster cardiac xenografts in rats. Transplantation Proceedings. June 1994, Vol. 26, No. 3, pages 1382-1383, see entire document. | 1-26 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
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|---|---|
| Date of the actual completion of the international search<br>20 JUNE 1996 | Date of mailing of the international search report<br>02 JUL 1996 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>RON SCHWADRON<br>Telephone No. (703) 308-0196 |

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# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/06804

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 39/395, 39/00, 38/00, 38/16, 38/17; C07K 16/00, 16/18, 16/28, 16/44, 16/46, 14/00, 14/47; C12N 5/00, 5/06, 5/10, 5/12; G01N 33/53; C07H 21/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/133.1, 135.1, 152.1, 172.1, 177.1, 800, 801, 810, 93.7; 435/7.1, 240.27, 320.1, 240.2; 514/2, 885; 530/387.1, 387.3, 388.2, 389.1, 827, 866, 867, 868; 536/23.53

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

424/133.1, 135.1, 152.1, 172.1, 177.1, 800, 801, 810, 93.7; 435/7.1, 240.27, 320.1, 240.2; 514/2, 885; 530/387.1, 387.3, 388.2, 389.1, 827, 866, 867, 868; 536/23.53